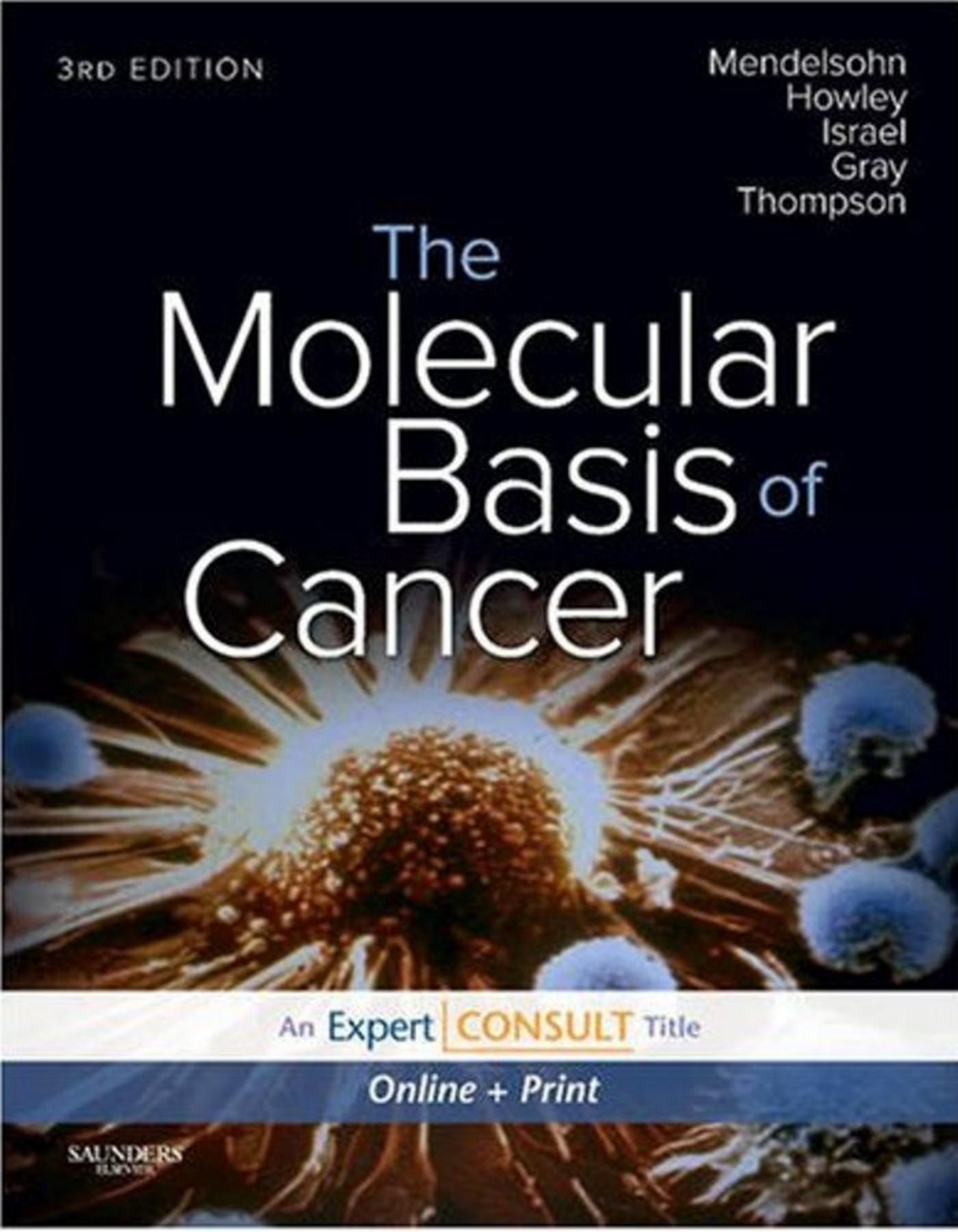


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THE MOLECULAR BASIS OF CANCER

978-1-4160-3703-3

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The Publisher

Previous editions copyrighted 2001, 1995

Library of Congress Cataloging-in-Publication Data

The molecular basis of cancer / [edited by] John Mendelsohn...[et al.]. – 3rd ed.
p. ; cm.

Includes bibliographical references and index.

ISBN 978-1-4160-3703-3

1. Cancer—Molecular aspects. 2. Carcinogenesis. I. Mendelsohn, John, 1936-
[DNLN: 1. Neoplasms—genetics. 2. Molecular Biology. QZ 202 M7176 2008]
RC268.5.M632 2008
616.99'4071—dc22

2007042086

Editor: Dolores Meloni
Developmental Editor: Kim DePaul
Project Manager: Mary Stermel
Design Direction: Louis Forgione
Marketing Manager: William Veltre

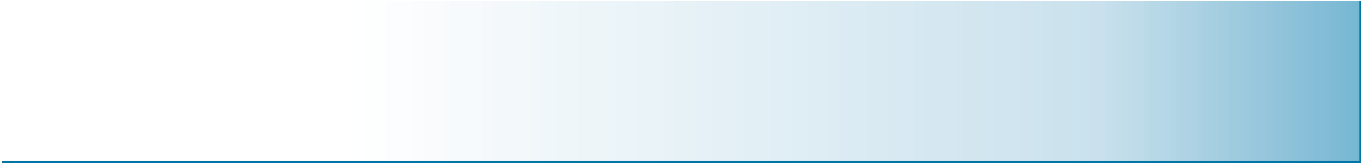
Printed in Asia

Last digit is the print number: 9 8 7 6 5 4 3 2 1

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This book is dedicated to our wives

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Preface

Molecular biology has revolutionized our understanding of the pathogenesis of cancer. Conversely, the study of malignancy has “transformed” our understanding of the molecular and genetic processes that govern the growth and proliferation of normal cells.

By 1995, our knowledge had expanded to the point that we felt it worthwhile to write a textbook describing *the molecular basis of cancer* for investigators, students, and providers of clinical care in a variety of disciplines. The aim in this third edition continues to be to explain, rather than to merely recount. Over the past decade, there has been a massive acceleration in the discoveries and observations that form the basis for understanding a disease which, until the 1990s, was thought about primarily in purely descriptive terms.

Five editors, selected for their expertise and for their reputations as educators, met to design a sequence of sections and chapters that would lead the reader from the basic genetic and molecular mechanisms of carcinogenesis to the molecular and biological features of cancer cell growth and metastasis, then to the new technologies that enable personalized risk assessment and early detection, followed by a description of the molecular abnormalities found in the common types of cancer, and finally to the molecular basis for new approaches to therapy.

The purpose of this textbook is not to detail the clinical manifestations of cancer or of its diagnosis and management with specific treatments; rather, it is to describe the scientific underpinnings that will enable clinicians and other professionals who manage cancer patients to better understand the disease and its therapy. This textbook will be of equal, or possibly greater, interest to laboratory and clinical investigators in biomedical research and to advanced students and trainees, who need to understand the molecular mechanisms that govern the functioning and malfunctioning of malignant cells. Although the chapters follow a sequence that moves from pathogenesis to therapy, each chapter stands alone in its treatment of the subject matter.

Cancer arises as a result of genetic and epigenetic alterations that either enhance, or diminish, the activities of pathways mediating normal cellular activities. Impaired capacity to repair genetic alterations can contribute to the likelihood that cells accumulate

these genetic abnormalities, leading to malignant transformation. Molecular influences from the environment around the cancer cell contribute importantly to the capacity of a genetically altered cell to become a tumor.

A remarkable lesson gained from cancer research is that the strategies utilized by widely divergent cell lineages to regulate growth and differentiation share common molecular pathways. The accumulated mutation or altered expression of genes critical for these pathways is a recurrent theme observed in many different tumor types. Tumors appear to select for genetic abnormalities that may be most advantageous for escape from normal regulatory mechanisms in their particular microenvironments.

Cancer is not merely a disorder of individual transformed cells. These cells grow into tumor masses and attract a blood supply, and they invade through surrounding tissues and metastasize.

Knowledge of the molecular basis of these complex processes is important for understanding the natural history of malignant disease and for designing treatment.

In the third edition of this textbook, we have introduced an entirely new section that describes the new laboratory technologies which enable investigators to determine the genetic and molecular abnormalities in cancers and to discover markers which enable prediction of risk and outcomes and selection of treatment.

The final section explores the process of clinical research and the new therapeutic agents against specific genetic, molecular and antigenic targets that are under investigation today.

What is most exciting today is the active dialogue between clinicians and laboratory scientists who share an interest in applying the new knowledge of genetics and molecular biology to the diagnosis, treatment, and prevention of disease. It is clear that during the next ten years we will have the opportunity to select treatments for clinical studies from among literally hundreds of new biological and chemical agents that target specific molecular irregularities in malignant cells. The knowledge we present in this textbook should supply a basis upon which new approaches to therapy can be evaluated by those interested in understanding and critically assessing the many new products of the biotechnology revolution.

The editors are delighted that we were able to recruit as contributing authors outstanding investigators who are excited about the challenge of presenting their areas of expertise in a textbook format. In many cases this has required more time and effort than they initially anticipated, and we are grateful for their dedication. We hope that we have come at least part of the way toward achieving what we set out to do. We have been assisted and encouraged

by the professionals at Elsevier, as well as the patient and ever-essential help of the secretaries in our offices.

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Cancer: A Genetic Disorder

Our understanding of the origins of cancer has changed dramatically over the past three decades, due in large part to the revolution in molecular biology that has altered the face of all biomedical research. Powerful experimental tools have been thrust into the hands of cancer biologists. These tools have made it possible to uncover and dissect the complex molecular machinery operating inside the single cell, normal and malignant; to understand its operations; and to pinpoint the defects that cause cancer cells to proliferate abnormally.

Three decades ago, at least three rival models of cancer's origins had substantial following among those interested in the roots of cancer. One model portrayed cancer as a disease of abnormal differentiation. According to this thinking, the changes in cell behavior that occur during the process of development run awry during tumor progression, causing cells to make inappropriate choices in moving up or down differentiation pathways. This concept of cancer's origins had important implications for the molecular origins of cancer: Since the process of differentiation involves changes in cell phenotype without underlying changes in the genome, this model suggested that cancer was essentially an epigenetic process—a change in cell behavior without an underlying change in its genetic constitution.

An alternative model was advanced by the virologists. By the early 1970s, a number of distinct cancer-causing viruses had been catalogued in various animal species and in humans. These ranged from the Rous sarcoma virus, whose discovery reached back to the first decade of the century, to Shope papilloma virus; Epstein-Barr virus; papova viruses like SV40 and polyoma virus; and a variety of retroviruses that infected various mammals and birds. The existence of these viruses suggested that similar agents operated to trigger human tumors. Such hypothetical human tumor viruses were thought capable of insinuating themselves into human cells and transforming them from a normal to a malignant growth state (1).

Yet another way of explaining cancer's origins was advanced by those who were impressed by the increasing connections being forged between carcinogens and mutagens. More than half a century of experiments had demonstrated the abilities of radiation and a vast array of chemicals to induce tumors in animals and occasionally in humans. Independent of this research, *Drosophila* and bacterial geneticists had documented the abilities of some of these carcinogenic agents to act as mutagens. The most influential of these experiments was to come from the laboratory of Bruce

Ames. In the mid 1970s, Ames described a correlation between the mutagenic potencies of various chemical compounds and their respective potencies to induce tumors in laboratory animals (2).

Ames' correlation (Figure 1-1) yielded the inference that the carcinogenic powers of agents derive directly from their abilities to damage genes and thus the DNA of cells. This strengthened the convictions of those who had long embraced the notion that cancer cells were really mutants, and that their abnormal behavior derived from mutant genes that they carried in their genomes. This model implied that such mutant genes arose through somatic mutations (i.e., mutations that occur in somatic tissues during the lifetime of an organism and alter genes that were pristine at the moment of conception).

This last model of cancer's origins would eventually dominate thinking; the other two models largely fell by the scientific wayside.

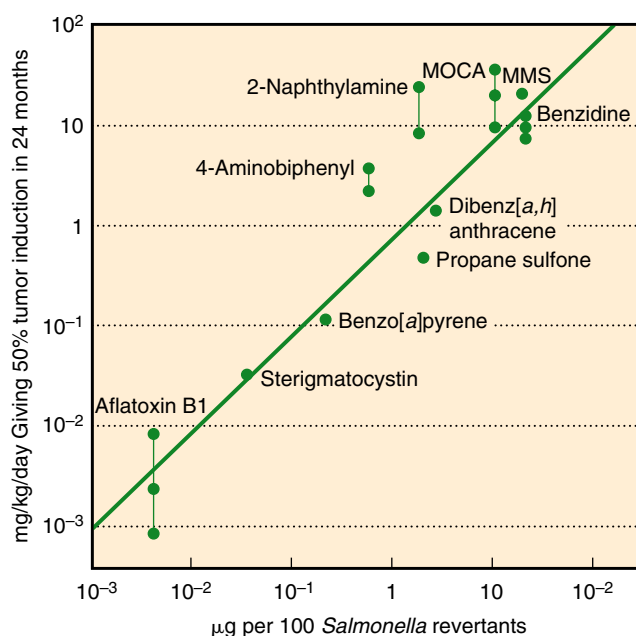


FIGURE 1-1 RELATIONSHIP OF CARCINOGENIC TO MUTAGENIC POTENCIES OF CHEMICAL COMPOUNDS. The ability to quantify both the mutagenic potencies of a variety of chemical compounds, measured in the Ames mutagenesis test, and to relate this to their carcinogenic potencies, as measured in laboratory rodents, allowed this graph and correlation to be made between the two mechanisms of action. (Adapted from Ref. 3.)

As the 1970s progressed, the search for tumorigenic viruses associated with most types of common human cancers bogged down. Human papilloma virus (HPV) clearly had strong associations with cervical carcinomas, Epstein-Barr virus (EBV) with Burkitt lymphomas in Africa and nasopharyngeal carcinomas in southeast Asia, and hepatitis B and C viruses (HBV, HCV, respectively) with hepatocellular carcinomas in east Asia. Together, these accounted for as much as 20% of tumors worldwide (4). But the remaining types of cancers, and thus the vast majority of human cancers arising in the Western world, had no obvious viral associations in spite of extensive attempts to uncover them.

The epigenetic model of cancer lost its attractiveness largely because an extensive array of mutant growth-controlling genes was discovered in the genomes of human tumor cells. So the focus shifted increasingly to genes, more specifically the genomes of cancer cells. And cancer genetics in the 1970s and early 1980s became a branch of somatic cell genetics—the genetics of cells and their somatically mutated genes.

The Discovery of Cellular Oncogenes

The notion that cancer cells were mutants should have motivated a systematic search for genes that suffered mutation during the development of tumors. Moreover, these mutant genes should possess another property: They needed to specify some of the aberrant phenotypes ascribed to tumor cells, including alterations in cell shape, decreased dependence on external mitogenic stimuli, and an ability to grow without tethering to a solid substrate (anchorage independence). The fact that viruses were not important causative agents of most types of human tumors generated another conclusion about these cancer-causing genes: They were likely to be endogenous to the cell rather than being imported into the cell from some external source. Stated differently, it seemed likely that these cancer genes were mutant versions of preexisting normal cellular genes.

In the 1970s, when this line of thinking matured, the experimental opportunities to test its validity were limited. The human genome, which harbored these hypothetical cancer genes, represented daunting complexity. Its vastness precluded any simple, systematic survey strategy designed to locate mutant growth-controlling genes within cancer cells. Indeed, even now, three decades later, the means for conducting effective systematic surveys for cancer genes do not exist. And so the discovery of cancer-causing genes—oncogenes as they came to be called—depended on a circuitous, indirect experimental strategy.

Ironically, it was tumor viruses, in the midst of being discredited as important etiologic agents of human cancer, that led the way to finding the elusive cancer genes. Varmus and Bishop's study of the Rous sarcoma virus (RSV) broke open the puzzle. Their initial agenda was to understand the replication strategy of this chicken virus. However, in the years after 1974, they focused their attentions to unraveling the mechanism used by RSV to transform an infected normal cell into a tumor cell.

Earlier work of others had indicated that a single gene, named *src*, carried the vital cancer-causing information present in

the viral genome. Accordingly, the Varmus and Bishop laboratory launched a research program to trace the origins of this virus-associated *src* oncogene. In fact, the origins of most viral genes were obscure, shrouded in the deep evolutionary past. It seemed that most viruses and thus their genes originated hundreds of millions of years ago, perhaps as derivatives of the cells that they learned to parasitize.

But as this team reported in 1976, the *src* gene behaved differently: it was a recent acquisition by the Rous virus. Many closely related retroviruses shared with RSV an ability to replicate in chicken cells and a very similar set of genes needed for viral replication. However, these other viruses lacked the *src* gene and the ability to transform infected cells into cancer cells, suggesting that the *src* oncogene carried by RSV was a relatively recent genetic acquisition. The Varmus–Bishop group soon traced the origins of the *src* gene to an unexpected source—a closely related gene that resided in the genome of normal chickens and, by extension, in the genomes of all vertebrates. They named this gene *c-src* (cellular *src*) to distinguish it from the *v-src* (viral *src*) oncogene carried by the virus (5).

The Varmus–Bishop evidence converged on a simple conceptual model. It explained all their observations and ultimately much more. The progenitor of RSV lacked the *v-src* gene but grew well in chicken cells. During one of its periodic forays into a chicken cell, this ancestor virus picked up a copy of the *c-src* gene and incorporated it into its own viral genome. Once *src* was present within the viral genome, this slightly remodeled gene—now *v-src*—was exploited by RSV to transform cells it encountered in subsequent rounds of infection.

This provided a testimonial to the cleverness and plasticity of retroviruses, which seemed able to capture and then exploit normal cellular genes to do their bidding. But another implication was even more important: The Varmus–Bishop work pointed to the existence of a normal cellular gene, the *c-src* gene, that seemed to possess a latent ability to induce cancer. This cancer-causing ability was unmasked when the *c-src* gene was abducted by the chicken retrovirus that became the progenitor of RSV (Figure 1-2).

The *c-src* gene was named a “proto-oncogene” to indicate its inherent potential to become activated into a cancer-causing oncogene. Within several years, it became clear that as many as a dozen other tumorigenic retroviruses also carried oncogenes, each of which had been abstracted from the genome of an infected vertebrate cell (6,7). Hence, there were many proto-oncogenes in the normal cell genome, not just *c-src*. Each seemed to be present in the DNA of a normal mammalian or avian host species, and by extension, present in the genomes of all vertebrates.

These discoveries were momentous because they demonstrated that normal cellular genes had the ability to induce cancer if removed from their normal chromosomal context and placed under the control of one or another retrovirus. Still, a key piece was missing from this puzzle. Retroviruses seemed to be absent from most, indeed from almost all, human tumors. Could proto-oncogenes ever become activated without direct intervention by a marauding retrovirus?

An obvious response was that proto-oncogenes might be altered by mutational events that did not remove these genes from their normal chromosomal roosts. Instead, these mutations

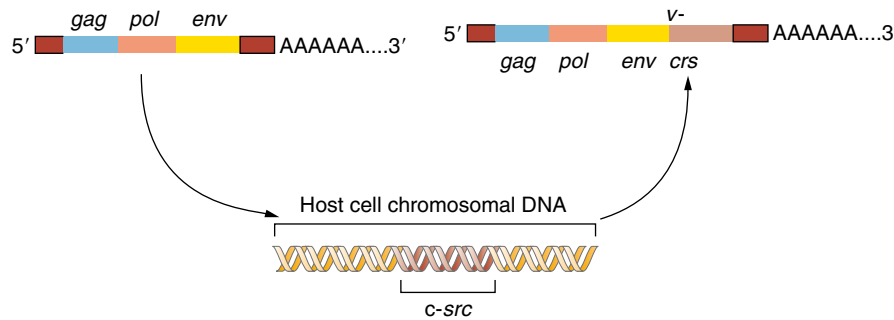


FIGURE 1-2 THE ORIGIN OF THE ROUS SARCOMA VIRUS *src* ONCOGENE. The acquisition of the *v-src* oncogene by a precursor of Rous sarcoma virus apparently occurred when an avian leucosis virus (ALV) lacking this oncogene infected a chicken cell and appropriated the cellular *c-src* proto-oncogene, thereafter carrying this acquired gene and exploiting it to transform subsequently infected cells.

would alter proto-oncogenes *in situ* in the chromosome by affecting either the control sequences or the protein-encoding sequences of these genes. This notion led to another question: If some proto-oncogenes could become activated by somatic mutations, such as those inflicted by chemical or physical carcinogens, would these be the same proto-oncogenes that were the targets of mobilization and activation by retroviruses?

In 1979 and 1980, answers came, once again from unexpected quarters. These newer experiments depended on the use of gene transfer, also known as transfection. The transfection procedure could be used to convey DNA, and thus genes, from tumor cells into normal recipient cells. The goal here was to see whether the transferred tumor cell DNA could induce some type of malignant transformation in the recipient cells. Success in such an experiment would indicate that the transferred gene(s) previously operated in the donor tumor cell to induce its transformation.

These transfection experiments succeeded (Figure 1-3). DNA extracted from chemically transformed mouse fibroblasts was able to induce normal mouse fibroblasts to undergo transformation (8). Retroviruses were clearly absent from both the donor tumor cells and the recipients that underwent transformation and so could not be invoked to explain the cancer-causing powers of the transferred DNA. Soon the identity of these transferred genes, which functioned as oncogenes, became apparent. They were members of the *ras* family of oncogenes, which had initially been discovered through their association with rodent sarcoma viruses

(7,9). These rodent retroviruses had acquired *ras* proto-oncogenes from normal rodent cells, much like RSV, which had stolen a copy of the *src* proto-oncogene from a chicken cell.

Unanswered by this was the genetic mechanism that imparted oncogenic powers to the tumor-associated *ras* oncogene, more specifically an H-*ras* oncogene. It soon became clear that the tumor-associated H-*ras* oncogene was closely related to, indeed virtually indistinguishable from, a normal H-*ras* proto-oncogene that was present in the genomes of all vertebrates. Still, the tumor-associated *ras* oncogene carried different information than the precursor proto-oncogene: The oncogene caused the malignant transformation of cells into which it was introduced, while the counterpart proto-oncogene had no obvious effects on cell phenotype. This particular puzzle was solved in 1982 with the finding that a H-*ras* oncogene cloned from a human bladder carcinoma carried a point mutation—a single nucleotide substitution—that distinguished it from its counterpart proto-oncogene (10–12). This genetic alteration, clearly a somatic mutation, sufficed to convert a normally benign proto-oncogene into a virulent oncogene.

Within months, yet other activated oncogenes were found in human tumors by using DNA probes prepared from a variety of retrovirus-associated oncogenes. The *myc* oncogene, initially associated with avian myelocytomatosis virus, was found to be present in increased gene copy number (i.e., amplified) in some human hematopoietic tumors (13); in yet others, *myc* was activated

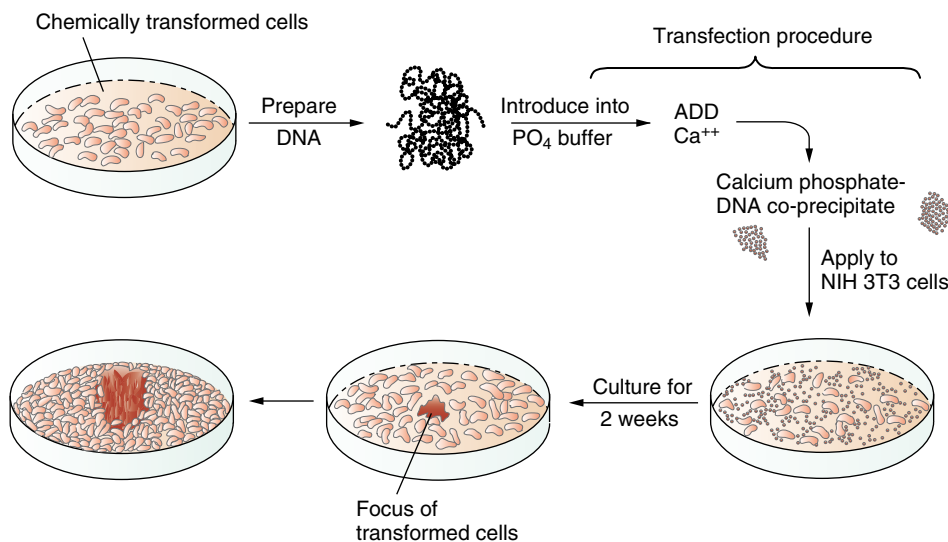


FIGURE 1-3 TRANSFECTION OF A CELLULAR ONCOGENE. The fact that the carcinogenicity of various chemical compounds was correlated with their mutagenicity suggested that cancer cells often carry mutant, cancer-inducing genes (i.e., oncogenes) in their genomes. This could be proven by an experiment in which DNA was extracted from chemically transformed mouse fibroblasts and introduced, via the procedure of transfection, into untransformed mouse fibroblasts. The appearance of foci of transformed cells in the latter indicated the transmission of a transforming gene from the donor to the recipient cells, indicating that chemical carcinogens could indeed generate a mutant, cancer-causing gene.

through a chromosomal translocation that juxtaposed its coding sequences with those of immunoglobulin genes, thereby placing the expression of the *myc* gene under the control of these antibody genes rather than its own normal transcriptional control elements (14). These discoveries extended and solidified a simple point: a common repertoire of proto-oncogenes could be activated either by retroviruses (usually in animal tumors) or by somatic mutations (in human tumors). The activating mutations involved either base substitution, amplification in gene copy number, or chromosomal translocation.

Multistep Tumorigenesis

The discoveries of mutant, tumor-associated oncogenes in human tumors led to a simple model of cancer formation. Mutagenic carcinogens entered into cells of a target tissue and mutated a proto-oncogene. The resulting oncogene then induced the now-mutant cell to initiate a program of malignant growth. Eventually, years later, the progeny of this mutant founder cell formed a large enough mass to become a macroscopically apparent tumor.

While satisfying conceptually, this simple model of cancer formation clearly conflicted with a century's worth of histopathologic analyses, which had indicated that tumor formation is really a multistep process, in which initially normal cell populations pass through a succession of intermediate stages on their way to becoming frankly malignant. Each of these intermediate stages contains cells that were more aberrant than those seen in the preceding steps. This body of observations persuaded many that the formation of a malignancy depended on a succession of phenotypic changes in the cells forming these various growths. Quite possibly, each of these shifts in cell phenotype reflected a change in the underlying genetic makeup of the evolving premalignant cell population. Such a multistep genetic model of tumor progression stood in direct conflict with the single-hit model of transformation that was suggested by the discovery of the point-mutated *ras* oncogene.

By 1983, one solution to this dilemma became apparent. In that year, experiments showed that a single introduced oncogene could not transform fully normal rat cells into ones that were tumorigenic. Two and maybe even more oncogenes seemed to be required to effect this conversion (15,16). For example, although an introduced *ras* oncogene could not transform normal embryo cells into tumor cells, the co-introduction of a *ras* plus a *myc* oncogene, or a *ras* plus an adenovirus E1A oncogene, succeeded in doing so. It appeared that such pairs of oncogenes collaborated with one another to induce the full malignant transformation of normal cells (Figure 1-4A). Moreover, this experiment suggested that human tumors carried two or more mutant oncogenes that collaborated with one another to orchestrate the many aberrant phenotypes associated with highly malignant cells.

Observations like these pointed to a new way of conceptualizing the multistep tumorigenesis long studied by the pathologists. It seemed plausible that each of the histopathological transitions arising during tumor development occurred as a consequence of a new mutation sustained in the genome of an evolving, premalignant

cell population (Figure 1-4B). According to this thinking, tumor development was a form of Darwinian evolution in which each successive mutation in a growth-controlling gene conferred increased proliferative potential and thus selective advantage on the cells bearing the mutant gene (17,18). Ultimately, a multiply-mutated cell bearing half a dozen or more mutant genes might exhibit all of the phenotypes associated with highly malignant cancer cells.

This mechanistic model was validated through the creation of transgenic mice. Cloned copies of mutant oncogenes, such as *ras* and *myc*, were introduced into the germ lines of mice. These transgenes were structured so that the oncogene was placed under the control of a transcriptional promoter that ensured expression of the resulting "transgene" in a specific tissue or developmental stage. Now the presence of a mutant oncogene in a particular tissue could be guaranteed through the actions of an appropriately engineered transgene rather than being dependent on the random actions of mutagenic carcinogens.

In one highly instructive group of experiments, a *myc* or a *ras* oncogene was placed under the control of the mouse mammary tumor virus transcriptional promoter, which guaranteed its expression in the mammary epithelium of the pregnant female mouse (19). As anticipated, these mice contracted breast cancer at extremely high rates. This demonstrated that mutant oncogenes were far more than markers of cancer progression; indeed, they could actually play a causal role in driving tumor pathogenesis.

Significantly, the transgenic mice did not contract cancer rapidly in their mammary tissue even though a mutant oncogene was implanted and expressed in virtually all of the epithelial cells of their mammary glands. Instead, their mammary carcinomas arose with several months' delay, indicating that a second (and perhaps third) alteration was required in addition to the activated transgene before mammary epithelial cells launched a program of malignant growth. The nature of this additional alteration(s) was not always clear, but almost certainly involved stochastic somatic mutations striking the mammary epithelial cells, creating mutant growth-controlling genes that collaborated with the transgene to trigger the outgrowth of malignant cell clones. In the years that followed, this work was extended to many types of human tumors, the cells of which were found to possess multiple mutant genes that contributed to tumor formation.

The Discovery of Tumor Suppressor Genes

The model of multistep tumorigenesis implied that a tumor cell carries two or more mutant oncogenes, each activated by somatic mutation during one of the stages of tumor development. However, experimental validation of this model initially proved to be difficult. Most attempts at detecting mutant oncogenes in human tumor genomes yielded a *ras* or perhaps an *myc* oncogene, but rarely were two mutant oncogenes found to coexist in the genomes of human tumor cells. This left two logical alternatives. Either the genome of a typical human tumor cell did not contain multiple mutated genes, as the multistep model of

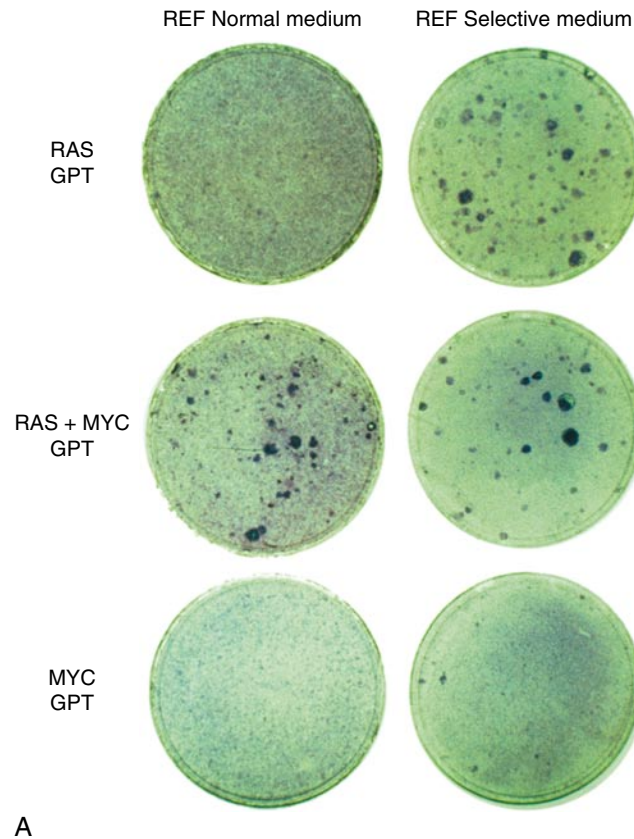
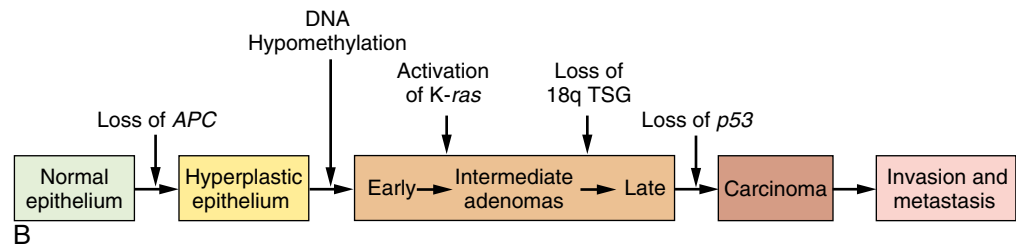


FIGURE 1-4 MULTISTEP TUMORIGENESIS *in vitro* AND *in vivo*. **A:** The ability of oncogenes to collaborate to transform cells *in vitro* was illustrated in this 1983 experiment in which neither a *ras* nor a *myc* oncogene was found able to induce foci when introduced into early passage rat embryo fibroblasts (REFs). However, when the two were introduced concomitantly, transformation ensued, as indicated by the appearance of foci. This suggested that tumor progression *in vivo* might involve a succession of mutations that created multiple collaborating cellular oncogenes. **B:** By 1989, analyses of the genomes of colonic epithelial cells at various stages of tumor progression revealed that the more progressed the cells were, the more mutations they had acquired. In fact, some of the indicated mutations involved inactivation of tumor suppressor genes, to be discussed below. (A: From Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 1983;304:596–602; B: Courtesy of B. Vogelstein, Johns Hopkins school of medicine).



cancer suggested, or there were indeed multiple mutated cancer-causing genes in tumors, but many of these were not oncogenes of the type that had been studied intensively in the 1970s and early 1980s.

In fact, there were candidate genes waiting in the wings. These others operated in a fashion diametrically opposite to that of the oncogenes: They seemed to prevent cancer rather than favoring it and came to be called tumor suppressor genes. Several independent lines of evidence led to the discovery and characterization of these genes.

Experiments using cell hybridization initiated by Henry Harris in Oxford provided the first indication of the existence of these suppressor genes (20). These cell hybridizations involved the physical fusion of two distinct types of cells that were propagated in mixed cultures. The conjoined cells would form a common hybrid cytoplasm and ultimately pool their chromosomes, yielding a hybrid genome.

Often these cell hybridizations involved the fusion of cells with two distinct genotypes. In some of these experiments, tumor cells were fused with normal cells. The motive here was to see which genome would dominate in determining the behavior of the resulting hybrids. Counter to the expectations of many, the resulting hybrid cells turned out, more often than not, to be nontumorigenic (21). This indicated that the genes present in the normal genome dominated over those carried in the cancer cell. In the language of genetics, the normal alleles were dominant, while the cancer cell-associated alleles were recessive. (More properly, the alleles present in the cancer cell created a phenotype that was recessive to the normal cell phenotype.)

This unanticipated behavior could most easily be rationalized by assuming that normal cells carried certain growth-normalizing genes, the presence of which was needed to maintain normal proliferation. Cancer cells seemed to have lost these genes, ostensibly through mutations that resulted in inactivated versions

of the genes present in normal cells. When reintroduced into the cancer cells via cell fusion, the normal alleles reimposed control on the cancer cells, restoring their behavior to that of a normal cell. In effect, these growth-normalizing genes suppressed the tumorigenic phenotype of the cancer cells and were, for this reason, termed “tumor suppressor genes” (TSGs).

In their normal incarnations, the TSGs seemed to constrain growth, unlike the proto-oncogenes which seemed to be involved in promoting normal proliferation. *Inactivated*, null alleles of TSGs were found in tumor cell genomes in contrast to the *hyperactivated* alleles of proto-oncogenes (i.e., oncogenes) found in these genomes.

The study of retinoblastoma, the childhood eye tumor, converged on these cell hybridization studies in a dramatic way. This work had been pioneered by Alfred Knudson who, beginning in the early 1970s, studied the genetics of this rare tumor. Knudson learned much by comparing the two forms of this cancer: sporadic retinoblastoma, which seemed to be due exclusively to accidental somatic mutations, and familial retinoblastoma, which appeared, like many familial cancers, to be due to the transmission of a mutated gene in the germ line.

Knudson’s analysis of the kinetics of retinoblastoma onset persuaded him that a common set of gene(s) operated to generate both kinds of tumors (21,22). Although the nature of these genes eluded him, their number was clear. Sporadic retinoblastomas seemed to arise following two successive somatic mutations affecting a lineage of cells in the retina. The triggering of familial retinoblastomas seemed to require only a single somatic mutation. Knudson speculated that in these familial tumors, a second mutated gene was required to trigger tumorigenesis and that this gene was already present in mutant form in all the cells of the retina, having been inherited in mutated form from a parent of the affected child.

For the cancer geneticist, Knudson’s most important concept was the notion that a retinal cell needed to lose two mutant genes before it was transformed into a tumor cell. Sometimes one of the two mutant null alleles was contributed by the germ line; more often, both genes arose through somatic mutation. But the nature of these genes and the mutations that recruited them into the tumorigenic process remained elusive. Finally, in 1979, karyotypic analysis of a retinoblastoma revealed an interstitial deletion in the q14 band of chromosome 13 (23). Later work revealed that this resulted in the loss of a gene, termed “*RB*.” Hence, one of the two mutational events needed to make a retinoblastoma involved the inactivation of an *RB* gene copy, in this particular case through the wholesale deletion of the chromosomal region carrying the *RB* gene.

By 1983, the nature of the second mutational event became clear: It involved the loss of the second, hitherto intact copy of the *RB* gene (24). Hence, the two mutational events hypothesized by Knudson involved the successive inactivation of the two copies of this gene. Suddenly, the need for two mutations became clear: The first mutation left the cell with a single, still-intact copy of the *RB* gene, which was able, on its own, to continue programming normal proliferation. Only when this surviving gene copy was eliminated from the cell genome did runaway proliferation begin (Figure 1-5).

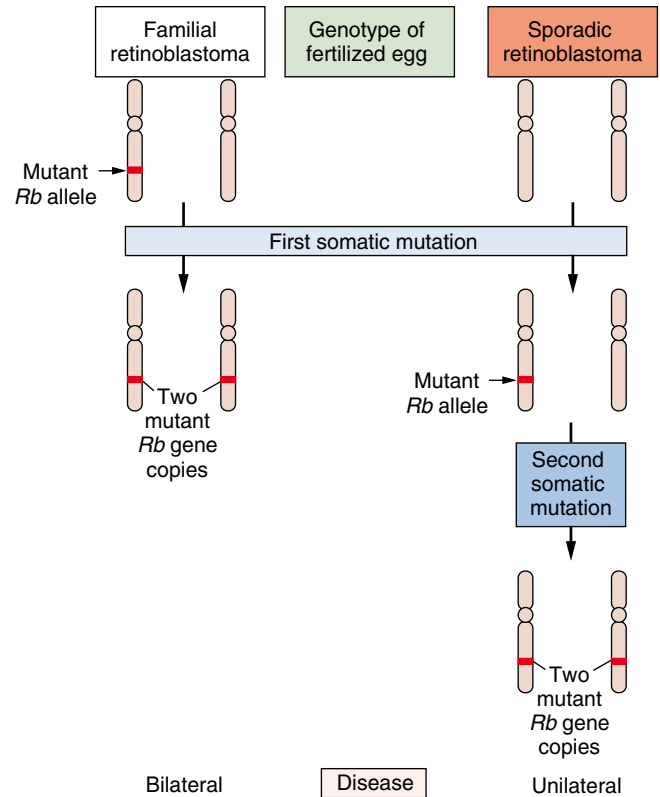


FIGURE 1-5 GENETICS OF RETINOBLASTOMA DEVELOPMENT. The development of retinoblastomas requires the successive inactivation of two copies of the chromosomal retinoblastoma (*Rb*) gene. In the case of familial retinoblastomas, one of the two copies of this gene is already mutated in one or another gamete and is transmitted to the offspring, who is therefore heterozygous at this locus in all cells of the body; subsequent loss, through somatic alterations, of the surviving wild-type gene copy leaves a retinal cell with no functional copies of this gene, enabling tumor formation to begin. In sporadic retinoblastomas, the conceptus is genetically wild-type; however, two successive somatic mutations occurring in a lineage of retinal precursor cells leaves some of these cells, once again, without functional *Rb* gene copies, and as before, permits retinoblastoma tumorigenesis to begin.

Thus, mutations that inactivate an *RB* gene copy create alleles that function recessively at the cellular level. Only when both wild-type alleles are lost through various mutational mechanisms does a retinal cell begin to behave abnormally.

The *RB* gene became the paradigm for a large cohort of similarly acting TSGs that suffer inactivation during tumor progression. These TSGs are scattered throughout the cell genome and act through a variety of cell-physiologic mechanisms to control cell proliferation (25). They are united only by the fact that they control proliferation in a negative way and that their loss permits uncontrolled cell multiplication to proceed.

The discovery of the *Rb* gene gave substance and specificity to the genes that Harris had postulated from his cell fusion experiments. Equally important, they opened the door to understanding a variety of familial cancer syndromes. In the case of *RB*, inheritance of a mutant, defective allele predisposes to retinoblastoma early in life with more than 90% probability. Inheritance of a defective allele of the *APC* TSG predisposes with high frequency to adenomatous polyposis coli syndrome and thus to colon cancer. The presence of a mutant *TP53* gene in the germ line leads to increased rates of tumors in a number of organ sites, including sarcomas

and carcinomas, yielding the Li-Fraumeni syndrome. More than two dozen heritable cancer syndromes have been associated with germ-line inheritance of defective TSGs (26,27).

In each case, the inheritance of a mutant, functionally defective TSG allele obviates one of two usually required somatic mutations. Because an inactivating somatic mutation represents a low-probability event per cell generation, the presence of an already-mutant inherited TSG allele enormously accelerates the overall kinetics of tumor formation. As a consequence, the likelihood of a tumor arising during the course of a normal life span is enormously increased.

The search for TSGs has been difficult, as their existence only becomes apparent when they are absent from a cellular genome. However, one peculiarity of TSG genetics has greatly aided the discovery of these genes. This involves the genetic mechanisms by which the second copy of a TSG is lost. In principle, two independent somatic mutations could successively inactivate the two copies of a TSG, thereby liberating a cell from the growth-constraining influences of this gene. However, each of these mutations normally occurs with a low probability—perhaps 10^{-6} per cell generation. The likelihood of both mutations occurring is therefore roughly 10^{-12} per cell generation, an extremely probability. (Actually, because cancer cell genomes become progressively destabilized as tumors develop, this probability is usually higher.)

In fact, evolving premalignant cell populations carrying a single, already-inactivated TSG copy often resort to another genetic mechanism to eliminate the second, still-intact copy of this TSG. They discard the chromosomal arm (or chromosomal region) carrying the still-intact TSG copy and replace it with a duplicated copy of the chromosomal region carrying the mutant, already-inactivated TSG copy. All this is achieved via the exchange of genetic material between paired homologous chromosomes.

The end result of these genetic gymnastics is the duplication of the mutant TSG copy. Thus, the TSG goes from a heterozygous state (involving one mutant and one wild-type gene allele) to a homozygous state (involving two mutant gene copies). Almost always, the chromosomal region flanking the TSG suffers the same fate. Consequently, known genes as well as other genetic markers within this flanking region that were initially present in a heterozygous configuration now become reduced to a homozygous configuration. This genetic behavior has motivated cancer geneticists to analyze the genomes of human tumor cells, looking for chromosomal regions that repeatedly suffer loss of heterozygosity (LOH) during tumor progression. Such LOHs represent presumptive evidence for the presence of TSGs in these regions whose second wild-type copies have been eliminated by LOH during the course of tumor development. Once such a region is localized to a chromosomal region, several of currently available gene molecular strategies can be exploited to further narrow the chromosomal domain carrying the TSG and ultimately to isolate the TSG through molecular cloning.

The existence of many dozen, still-unknown TSGs is suspected because of the documented LOH affecting specific chromosomal regions of various types of human tumor cells. The effort to identify and clone these genes is being greatly facilitated by the fruits of the Human Genome Project. Nonetheless, the successful

identification and cloning of a significant cohort of TSGs have already provided one solution to a major puzzle posed in the preceding sections. As mentioned, while human tumors cells were hypothesized to carry a number of distinct, mutated growth-controlling genes, most tumors appeared to carry only a single activated oncogene. We now realize that many of the other targets of mutation during tumor progression are TSGs. Their inactivation collaborates with the activated oncogenes to create malignant cells and thus tumors. In the widely cited study of human multistep tumor progression—that described in colonic tumors by Vogelstein and his co-workers—the mutation of a *K-ras* oncogene is accompanied by mutations of the *APC* and *TP53* TSGs and a third TSG that maps to chromosome 18 (28). This evidence, together with the wealth of genetic studies reported subsequently, indicate that TSGs are inactivated even more frequently than oncogenes are activated during the course forming many types of human tumors. Importantly, the inactivation of TSGs often phenocopies the cell-biological effects of oncogenes. This means that the inactivation of TSGs is as important to the biology of tumor progression as oncogene activation.

Unexpectedly, the discovery of TSGs also made it possible to understand how a variety of DNA tumor viruses succeed in transforming the cells that they infect. Unlike retroviruses, these DNA viruses carry oncogenes that have resided in their genomes for millions, likely hundreds of millions, of years. Any connections with antecedent cellular genes, to the extent they once existed, were obscured long ago by the extensive remodeling that these oncogenes underwent while being carried in the genomes of the various DNA tumor viruses. Independent of their ultimate origins, it was clear in the 1980s that the oncogenes (and encoded oncoproteins) were deployed by DNA viruses to perturb key components of the normal cellular growth-controlling circuitry. However, the precise control points targeted by these viral oncoproteins remained obscure.

In the late 1980s, we learned that a number of DNA tumor virus oncoproteins bind to the products of two centrally important TSGs, pRB and p53 (29,30). For example, the large T oncoprotein of SV40 binds and sequesters both the p53 and pRB proteins of infected host cells; the E6 and E7 oncoproteins of human papilloma viruses target p53 and pRb, respectively. As a consequence, a virus-infected cell is deprived of the services of these two key negative regulators of its proliferation. Indeed, these virus-mediated inactivations closely mimic the state seen in many nonviral tumors that have been deprived of pRB and p53 function by somatic mutations striking the TSGs specifying these two proteins. So the transforming mechanisms used by these viruses could be rationalized by referring to the same genes and proteins that were known to be inactivated by mutational mechanisms in many types of spontaneous, nonviral human tumors. Importantly, these findings reinforced the notion that a single, central growth-regulating machinery operating in all types of cells suffers disruption by a variety of ostensibly unrelated genetic mechanisms, leading eventually to the formation of cancers.

The activation of oncogenes and the loss of TSGs together explain many of the phenotypes that one associates with cancer cells. These cells are able to grow without attachment to solid

substrate, the aforementioned phenotype of anchorage independence, and they are able to grow on top of one another, which is manifested in culture as the loss of contact inhibition. Moreover, when compared with normal cells, cancer cells exhibit a greatly reduced dependence on mitogens and an ability to resist the anti-proliferative effects of growth-inhibitory signals, such as those conveyed by TGF- β . Alterations of oncogenes and tumor suppressor genes can be invoked to explain these neoplastic cell traits.

Arguably the most interesting trait of cancer cells is their ability to resist a variety of stimuli and stresses that would cause normal cells to activate the cell-suicide program termed “apoptosis.” The fact that virtually all tumor cells have developed various types of resistance to apoptosis indicates that severe pro-apoptotic stresses are experienced repeatedly as normal cells evolve progressively toward a malignant phenotype, and that an ability to resist these stresses is strongly selected during this evolution. Thus, changes in the complex array of genes that control entrance in the apoptotic program are frequently demonstrable within tumor cells. While these genes are specialized in regulating a discrete cancer cell phenotype (apoptosis), they behave operationally like oncogenes and TSGs (i.e., the activation of some of these confers a resistance to apoptosis as does the inactivation of others).

Guardians of the Genome

As mentioned previously, the somatic mutations that activate oncogenes or inactivate TSGs are relatively rare events in the life of a cell, occurring perhaps at a rate of 10^{-6} per cell generation. This low mutation frequency represents an important barrier to the development of neoplasia (31). If cells require multiple mutations to progress to a fully malignant growth state, the probability of the entire constellation of mutations occurring within a cell lineage during a normal human life span is extremely low. This provides a partial explanation for the fact that humans develop relatively few cancers during life spans in which the cells in our bodies undergo more than 10^{16} divisions, each of which represents an opportunity for a genetic disaster.

As described earlier, the inheritance of a mutant growth-controlling gene obviates one of the normally required, rare somatic mutational steps. In doing so, it allows a population of premalignant cells to leapfrog over one of the barriers that usually block its progression toward malignancy. The consequence is the greatly increased risk of certain tumors that characterizes familial cancer syndromes.

But there is at least one other route by which this multistep tumor pathogenesis can be accelerated: If the rate of gene mutation per cell generation is greatly increased, the time required for a population of cells to surmount all of the mutational hurdles and progress to full-blown malignancy will be correspondingly reduced. As a consequence, the probability of cancer striking during a normal life span will be greatly increased.

Xeroderma pigmentosum (XP) is the most thoroughly studied of the inborn cancer susceptibility syndromes that are attributable to greatly increased mutational frequency. Those

suffering from XP show abnormally high sensitivity to ultraviolet (UV) radiation, which evokes squamous cell skin carcinomas and melanomas at exposed sites at a high rate. Like the rest of us, patients with XP sustain large numbers of mutational events in their skin cells created by UV photons. In the skin cells of most humans, the pyrimidine dimers created by UV radiation are quickly excised from the damaged DNA and the initial, wild-type nucleotide sequence is restored, thereby erasing all traces of the mutation; this removal of DNA lesions is achieved by a cohort of DNA repair proteins that are specialized to effect this particular alteration of DNA structure. (In the event that skin cells exhibit widespread genomic damage that overwhelms the ability of its DNA repair apparatus to restore normal genome sequence, the cell may opt for another response, apoptosis, as discussed in subsequent sections.) In the patient with XP, one or another essential component of this specialized DNA repair apparatus is absent or defective (32). As a consequence, altered DNA sequences are transmitted to the progeny of the initially irradiated cell, resulting in large numbers of mutations in their genomes. Hence, the effective mutation rate (the number of initially induced mutations minus those that are repaired) increases enormously.

XP represented only the first of the familial cancer syndromes that has been attributable to defective DNA repair. In this particular syndrome, mutational damage is inflicted by an exogenous mutagen—UV radiation. We now know that a variety of other familial cancer syndromes are also attributable to defects in one or another component of the complex apparatus that maintains the integrity of our genome. In many of the more-recently characterized cancer syndromes, the initial mutational damage is of endogenous origin, being inflicted by malfunctioning of normal cellular processes, including the mutations that result from mistakes in DNA replication and from the actions of endogenously generated mutagens, such as reactive oxygen species.

The ataxia telangiectasia syndrome, which includes, among its presentations, the development of certain tumors, is also due to defective DNA repair (32,33). In hereditary nonpolyposis colon cancer (HNPCC), the apparatus that recognizes recently made mistakes in DNA replication, often termed the “mismatch repair apparatus,” is defective (34,35). At least four different inherited subtypes of HNPCC have been described; each of these is due to defects in one or another component of the complex multi-component system that recognizes and erases DNA copying mistakes and other lesions that are occasionally inflicted on the cell genome. In the cells of patients with HNPCC, one sees widespread genomic instability, the direct results of this defective DNA repair. The resulting genetic damage seems to affect all genes with equal frequency, and thus the target proto-oncogenes and TSGs that participate in the formation of non-HNPCC colon cancers. As a consequence, the entire multistep process of colon cancer progression is greatly accelerated. Unexplained at present is why this genetic defect specifically afflicts the colon rather than causing elevated rates of cancer incidence in many sites throughout the body.

Many familial breast cancers have recently been associated with inheritance of mutant versions of the BRCA1 and BRCA2 genes (36). These were initially thought to be TSGs, but the

peculiar behavior of the mutant alleles of these genes suggested otherwise. Mutant alleles of the *BRCA1* and *BRCA2* genes were found to be inherited in the germ lines of affected individuals; however, sporadic mammary tumors rarely showed mutant alleles. Recent biochemical and cell biological experiments suggest that both these genes specify proteins that participate in the repair of double-strand DNA breaks. It remains unclear why the inheritance of defective alleles of either of these genes predisposes individuals specifically to breast and ovarian tumors.

There is increasing evidence that a breakdown of DNA repair capability accompanies the formation of the great majority of human tumors. These losses may occur through somatic mutation of DNA repair genes or, perhaps more frequently, through epigenetic mechanisms, such as DNA methylation (see below), that succeed in repressing the expression of these repair genes, thereby depriving cells of the vital functions encoded by these genes.

Epigenetic Mechanisms Leading to Loss of Gene Function

As described previously, the functions of two major classes of cellular genes are lost during the course of tumor progression—TSGs and DNA repair genes. It is highly likely that the development of most human tumors depends on these losses. Moreover, the portrayal of cancer as a genetic disorder, as developed in the preceding sections, would suggest that these genes and their vital functions are lost through various mechanisms of somatic mutation. After all, mutations are by definition heritable, and thus the progeny of a cell that has initially acquired growth advantage through some somatic mutation will be similarly benefited, leading to the progressive expansion of clones of such mutant cells.

Following this logic, the phenotypic changes that occur during the course of tumor progression need to be heritable. In fact, there is a mechanism of heritability that does not depend on genetic alterations (i.e., on alterations of nucleotide sequence in a cell's genome). This mechanism depends on the methylation of the cytidine residues present in CpG dinucleotide sequences that are found in proximity to the promoters of various genes. Such methylation often results in major shifts in the configuration of nearby chromatin, and in the shutdown of expression of nearby genes—the process of transcriptional repression.

When a DNA segment containing a methylated CpG is replicated, the complementary CpG in the newly synthesized daughter DNA strand is initially unmethylated. However, soon after this daughter strand is formed, “maintenance” DNA methylases recognize the hemi-methylated DNA and attach a methyl group to the recently formed CpG residue, thereby ensuring that both CpGs are now methylated (Figure 1-6). This scheme ensures that DNA methylation events, and thus associated repression of certain genes, can be transmitted from parent to daughter cells with high fidelity. Hence, genes may be inactivated in a heritable fashion without any change in their nucleotide sequence.

In fact, the mechanisms that control DNA methylation result in the inactivation of genes at higher rates per cell generation than those involving somatic mutations. This leads to the obvious conclusion that the functions of TSGs and DNA repair genes are likely to be lost more frequently through DNA methylation than mutation, a notion that is borne out by extensive studies of the genomes of human tumor cells (37). Indeed, it now seems likely that individual tumor cell genomes bear many dozens, if not hundreds, of methylated genes. Most of these genes are likely methylated as a consequence of the relaxed controls on DNA methylation that seem to operate within cancer cells; most of such genes are bystanders (i.e., their loss is not functionally important for the cancer cell phenotype and their loss has not conferred selective advantage on the cells that carry them). However, a number of key TSGs and DNA repair genes have been found to be methylated frequently in various types of human cancer cell genomes, and it is clear that the loss of gene function through promoter methylation is as effective in driving tumor progression as the somatic mutations that have been described extensively here. Hence, cancer pathogenesis is a disorder of genes and gene function, but does not always depend on genetic alterations, since the epigenetic mechanism of promoter methylation contributes as frequently, if not more frequently, to tumor formation.

Immortalized Proliferation

Yet another phenotype of cancer cells—their ability to grow and divide indefinitely—depends on changes in DNA structure and is, in this sense, a genetically determined trait. This unlimited proliferative ability, often termed “cell immortality,” stands

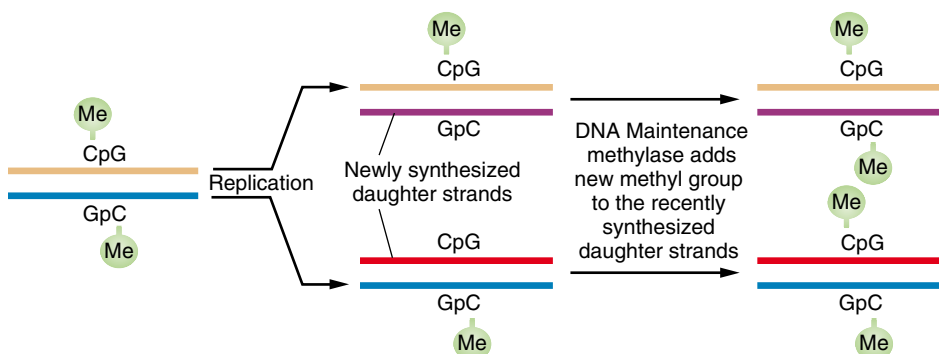


FIGURE 1-6 PERPETUATION OF CpG METHYLATION FOLLOWING DNA REPLICATION. When DNA methylated at CpG residues is replicated, the newly formed daughter strands initially lack methyl groups on the CpG sites complementary to those methylated sequences in the parental DNA strands. However, shortly after replication, maintenance methylases add methyl groups to the newly synthesized CpG sites, ensuring the transmission of the methylated state from one cell generation to the next. Such methylation is often associated with the repression of gene transcription.

in stark contrast to the limited proliferative ability of normal cell populations. Thus, when placed into culture, many types of cancer cells are able to proliferate indefinitely, in contrast to the behavior of normal cells, which cease proliferation after a limited, ostensibly predetermined, number of doublings. This phenomenon of finite replicative potential suggests the workings of some type of generational clock that tallies the number of cell divisions through which cell lineages have passed since they resided in the early embryo and then informs cells in these lineages when their allotment of doublings has been exhausted. In response to this alarm, cell populations become “senescent,” and if they overcome or circumvent senescence, will multiply further until they enter into a state of “crisis,” in which almost all of them die (38,39).

This limitation on replicative potential would seem to represent an important antineoplastic barrier erected by the organism. By limiting the number of successive replicative doublings its component cells may undertake, the organism erects a high barrier to the unlimited expansion of preneoplastic cell clones. Cancer cells must surmount this barrier to succeed in their agenda of unlimited growth and the formation of macroscopic tumors.

In fact, very different mechanisms govern the timing of the entrance of cell populations into senescence and into crisis. The senescence observed with cultured cells appears to be determined, in large part, by the conditions of their propagation *in vitro*. By necessity, the protocols developed for culturing cells create conditions that differ dramatically from those operating within living tissues. These discrepancies derive from the contents of the culture medium as well as the oxygen tensions experienced by cells within tissue culture incubators. As a consequence, cells suffer substantial physiologic stress when placed into culture, and cumulative cell-physiologic stress seems to be a major, if not *the* major, determinant of the triggering of senescence.

The mechanisms governing entrance into crisis are different and do involve, quite directly, the cell genome, more specifically the telomeres at the ends of all chromosomes. Evidence accumulated in recent years points to the telomeres as the molecular devices that tally cell generations and govern entrance into crisis. The ends of the telomeric DNA are not copied completely during each cycle of DNA replication due to an intrinsic limitation in the DNA polymerases responsible for the bulk of DNA replication. In addition, the ends of telomeric DNA are susceptible to the actions of exonucleases, which contribute to further erosion of telomeric DNA length. As a consequence, the telomeres shorten progressively as cell lineages pass through repeated cycles of growth and division (Figure 1-7). In normal cell lineages, this shortening eventually results in critically truncated telomeres. Without the protective effects of the telomeres, chromosomes undergo end-to-end fusion with resulting karyotypic instability and cell death. Hence, the progressive shortening of telomeres represents an effective molecular device for counting cumulative generational doublings (38,39).

Cancer cell populations must overcome this limitation on their proliferation in order to expand and generate macroscopic tumors. They do so by activating expression of the telomerase enzyme, which is able to restore and maintain telomeric DNA

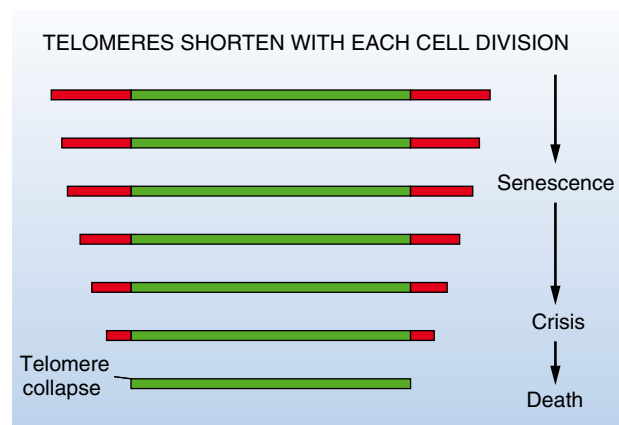


FIGURE 1-7 TELOMERE EROSION AND ENTRANCE INTO CRISIS. In the absence of active intervention by the telomerase enzyme, the telomeres of human chromosomes shorten progressively during each round of cell growth and division, eventually losing so much length that they can no longer subserve their normal function of protecting the ends of chromosomal DNA from end-to-end fusions with other chromosomes. This leads to massive cell death, termed “crisis,” and occasionally, the emergence of a rare variant that has indeed acquired telomerase expression and is accordingly now able to repair and maintain telomeric DNA and thus telomeres. (While the onset of senescence is indicated here as also being triggered by telomere shortening, it appears that it is largely due to cumulative cell-physiologic stresses sustained by cells both *in vitro* and *in vivo*.)

length, thereby reversing the effects of telomere erosion. Telomerase activity is detectable in almost all ($\approx 90\%$) human tumors but is present at low or undetectable levels in the corresponding normal tissues. Accordingly, the genes that allow telomerase activation during tumor progression represent additional important genetic elements that are affected during the development of almost all human tumors. Importantly, however, the human telomerase gene, *hTERT*, is not itself the target of mutation. Instead, its expression is induced by a complex array of *trans*-acting transcriptional regulators, the MYC oncoprotein being one of these.

The critical contribution of telomerase to tumorigenesis is illustrated most dramatically by the protocols that enable the experimental transformation of normal human cells into tumor cells. By adding the *hTERT* gene to a cocktail of other introduced oncogenes, a variety of normal human cells can be converted to a tumorigenic state, as judged by their behavior following implantation into appropriate host mice (40). The *hTERT* gene clearly affords such cells the ability to proliferate indefinitely; without its actions, cells fail to proliferate extensively *in vitro* and to form tumors *in vivo*.

Nongenetic Mechanisms Accelerating Multistep Tumor Progression

The descriptions of tumorigenesis, as developed here, lead to the notion that the functioning of normal cell genomes is progressively degraded by mutagenic mechanisms, promoter methylation, and telomerase erosion, and that these mechanisms conspire to drive forward multistep tumor progression. An obvious corollary is that exposure to high levels of mutagenic agents is likely to serve as a major

agent that stimulates human tumor formation. Indeed, since the initial experiments of Bruce Ames, such logic has inspired the search for the mutagens that are responsible for instigating human cancers.

In truth, with some notable exceptions, the search for the mutagenic carcinogens that drive human cancer pathogenesis has failed (41). Tobacco smoke, with its high levels of mutagens, is clearly responsible for almost one third of human cancers. In addition, the heterocyclic amine mutagens created by the cooking of red meat at high temperatures are attractive candidates for the agents causing many colon and possibly prostate cancers.

In general, however, the carcinogens responsible for most human cancer incidence have eluded identification, apparently because they do not function as mutagens. Instead, it has become increasingly apparent over the past two decades that the major determinants of human cancer incidence are various agents and conditions that operate as “tumor promoters.” Thus, as illustrated by the classic experiments involving mouse skin cancers, tumor “initiators” are responsible for triggering the first step of multistep tumorigenesis by mutating certain target genes (e.g., *H-ras*), while promoters are responsible for driving the clonal expansion of already-initiated tumor cells, doing so through mechanisms that do not involve genetic damage. It seems increasingly likely that most of the determinants of human cancer incidence operate as promoters.

Possibly the most important promoting mechanisms involve chronic inflammation of tissues, and the associated release of growth-stimulating factors by the irritated tissue. Moreover, many of the dietary determinants of tumor incidence would seem to function as promoters rather than as mutagenic initiators. If these notions are sustained by future research, this will mean that a complete understanding of cancer pathogenesis at the molecular level will require detailed elucidation of these nongenetic, tumor-promoting mechanisms.

Invasive and Metastatic Behaviors

In many individuals, the endpoint of multistep tumor progression involves, unfortunately, the acquisition by cancer cells of the ability

to invade and to metastasize from the primary tumor to distant sites in the body—the manifestations of high-grade malignancy. Indeed, the metastases that are spawned by malignant tumors are responsible for 90% of cancer-associated mortality.

The formation of metastases is the result of a complex, multistep process that is often termed the invasion-metastasis cascade (Figure 1-8). Thus, cancer cells in the primary tumor acquire the ability to invade adjacent tissue, enter into the vessels of the blood and lymphatic systems (intravasation), travel in these channels to distant sites in the body, escape from these vessels (extravasation) into nearby tissues, and establish small tumor colonies (micrometastases) in these tissues. On occasion, the cells forming a micrometastasis will acquire the ability to proliferate vigorously, resulting in the formation of a macroscopic metastasis—the process termed “colonization.”

The complexity of the invasion-metastasis cascade rivals that of the multistep process that leads initially to the formation of a primary tumor. This suggests, in turn, that cancer cells within a primary tumor must suffer a significant number genetic alterations to acquire the ability to complete this cascade. Another alternative has presented itself, however, as the result of research on the malignant behavior of carcinoma cells. This alternative mechanism involves the actions of genes that are normally involved in programming certain key steps of early embryonic morphogenesis. In such steps of embryogenesis, epithelial cells, which are normally immobilized in various layers, undergo a profound change in their differentiation program and acquire many of the phenotypes of mesenchymal cells, including motility and invasiveness. This transdifferentiation program is termed the “epithelial–mesenchymal transition” (EMT).

As many as half a dozen transcription factors acting during various stages of early embryogenesis are capable of programming EMTs. These transcription factors have names like Snail, Slug, Twist, Goosecoid, and SIP-1. Each of these is able to act pleiotropically to program an EMT, and thereby is able to cause the repression of epithelial genes and the induction, in their stead, of mesenchymal genes. Increasing experimental evidence indicates that carcinoma cells exploit these early embryonic genes to execute most of the steps of the invasion–metastasis cascade (42,43).

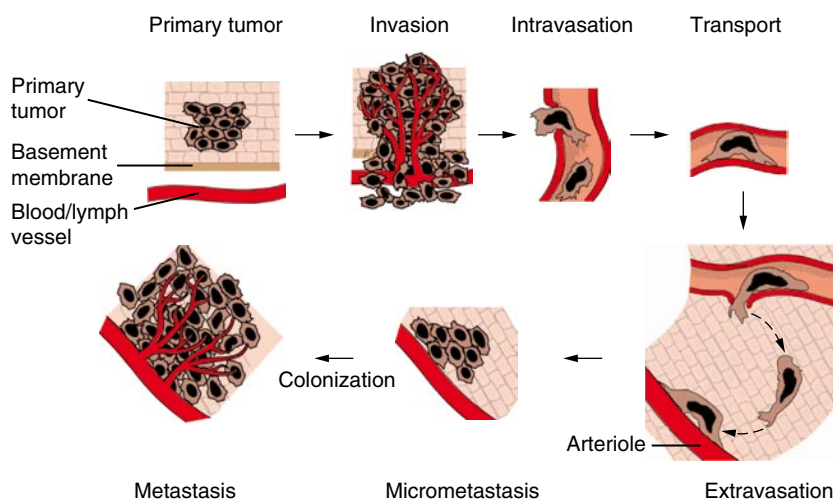


FIGURE 1-8 THE INVASION–METASTASIS CASCADE. The invasion–metastasis cascade is a complex, multistep process through which cancer cells must pass in order to launch macroscopic tumor colonies at distant sites. These steps are executed relatively inefficiently, resulting in vast numbers of cells being disseminated from primary tumors with only a small number of cells being able to eventually form macroscopic metastases.

Expression of these embryonic genes seems to be induced by contextual signals that these carcinoma cells experience in the tumor microenvironment and that originate in the tumor-associated stroma. For example, TGF- β impinging on certain cancer cells is able to elicit the expression of several of the transcription factors that are capable, in turn, of programming an EMT. This suggests that the EMT program, and the enabling of the invasion–metastasis cascade, occurs because of a collaboration between the genotype of cancer cells and the contextual signals that these cells receive from the nearby microenvironment, more specifically from the activated stroma that is present in many primary tumors. Moreover, it suggests that certain carcinoma cell genotypes render these cells responsive to such stromal, EMT-inducing signals, while other genotypes leave the cancer cells unresponsive, indeed refractory to these signals; our understanding of these genotypes is still fragmentary.

The discovery of these embryonic transcription programs and their resurrection by carcinoma cells greatly simplifies our conceptualization of the late stages of malignant progression. Rather than needing to acquire a number of distinct mutations to execute the various steps of the invasion–metastasis cascade, the genotypes of certain primary cancer cells allows them, in response to stromal signals, to activate long-dormant cell biological programs—EMTs. Once activated, this program seems to enable a carcinoma cell to complete most of the steps of the invasion–metastasis cascade. However, the last step—colonization—appears to involve an adaptation to the novel tissue microenvironment in which disseminated carcinoma cells have landed; such adaptation would not seem to be found among the multiple powers of the EMT program and would seem to acquire yet other changes that remain poorly understood.

Interestingly, the carcinoma cells forming a metastasis often recapitulate the histopathological appearance of the primary tumor, including its distinctive epithelial cell sheets and ducts. This would seem to be at variance with the notion that in order to metastasize, carcinoma cells must shed their epithelial characteristics and acquire, instead, mesenchymal ones. It seems plausible, however, that once carcinoma cells have disseminated and landed in distant tissue sites, they no longer encounter the mix of signals that were released by the activated stroma of the primary tumor and that led initially to their passing through an EMT. This new tissue microenvironment may therefore allow these cells to revert, via a mesenchymal–epithelial transition (MET) to the epithelial phenotype of their progenitors in the primary tumor, thereby generating once again epithelial histomorphology. Importantly, while passage through a partial or complete EMT may explain the malignant behavior of many carcinoma cells, it is less clear how tumors of other tissue origins, namely those arising in neuroectodermal, mesenchymal, and hematopoietic tissues, acquire these aggressive traits. The mechanisms enabling invasive and metastatic behaviors in these other neoplastic cell types remain elusive.

Other Phenotypes of Neoplasia

Many of the phenotypes of cancer cells are not readily explained by alterations in their proto-oncogenes and TSGs. Cancer cells

acquire other aberrations that favor their growth in the complex environments of living tissues. Included among these is their ability to recruit blood vessels into tumor masses—the process of angiogenesis (44)—and, quite possibly, their ability to evade and overwhelm immune defenses (45).

The process of tumor angiogenesis (Figure 1-9), like the EMT, involves a complex array of heterotypic interactions between cancer cells and their mesenchymal microenvironment. Indeed, this neoangiogenesis has become a subject of intensive investigation over the past decade, in part because the demonstrated dependence of tumors on vascularization represents an attractive target for therapeutic intervention through the creation and implementation of various antiangiogenic therapies. Thus, without adequate vascularization, cancer cells are limited to forming tumors smaller than 1 mm in diameter.

The processes of neovascularization depend on the heterotypic interactions of cancer cells with circulating endothelial precursor cells and with existing endothelial cells in the nearby stroma. Moreover, other regulators of this process include macrophages, myofibroblasts, and neutrophils, which may collaborate with the cancer cells to release angiogenic signals and thereby recruit endothelial cells and induce them to construct microvasculature. In addition, pericytes, which form the outer wall of most microvessels, must be recruited to ensure the assembly of well-constructed microvessels.

The role of the immune system in defending against the formation of various human tumor types remains a matter of great

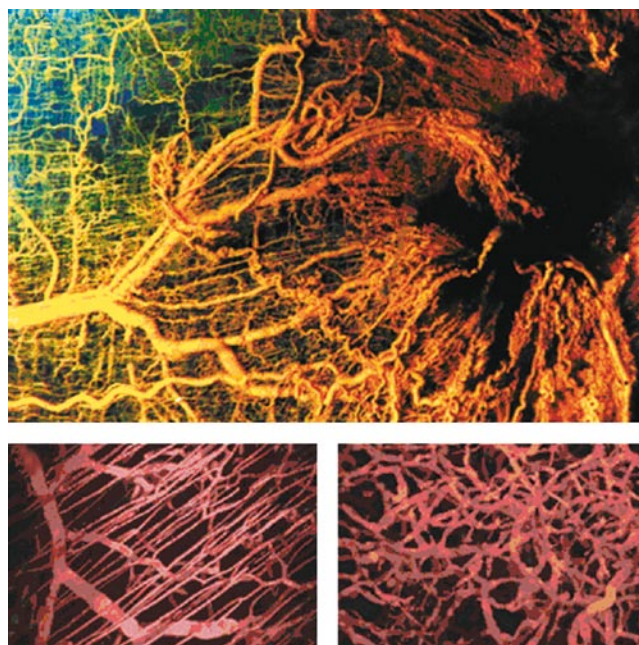


FIGURE 1-9 TUMOR ANGIOGENESIS. (Top) As tumors grow, they develop large networks of blood vessels through the process of angiogenesis. See tumor (black mass, right side) that has attracted blood vessels growing into it from adjacent normal tissue (left side). (Bottom) As is the case with most tumor-associated neovasculature, the new vessels developed here are tortuous and often end in dead ends, in contrast to the normal vasculature seen here (left side). (Top Courtesy of L. Heiser and R. Ackland, University of Louisville. Bottom images from Jain RK, *Nature Med* 2003;9 685–693, with permission.)

contention. Actually, in the case of virus-induced cancers, the protective role of the immune system is no longer debated, because of the abundant evidence that immunocompromised individuals suffer dramatically increased rates of virus-induced malignancies, including Kaposi sarcoma, human papillomavirus-induced squamous cell carcinomas, and certain types of Epstein-Barr virus-induced hematopoietic disorders. In all of these cases, these functions can be readily rationalized by invoking the known antiviral effects of the immune system.

More challenging, however, are the actions of the immune system in reducing the incidence of tumors of nonviral etiology, which constitute more than 80% of the total tumor burden in the population. In these cases, it has been unclear how the immune system can recognize tumor cells as being of foreign origin and proceed to attack and eliminate them. That such attack often occurs is clear, however, as evidenced by the several-fold increased incidence of a variety of common tumors in patients who are immunocompromised for various reasons, largely involving the preservation

of organ transplants. This phenomenon provides hope that the immune system is indeed capable of recognizing and attacking nonviral tumors and that its powers can be exploited to serve as antitumor therapeutic modalities.

The molecular genetic paradigm described here has allowed us to understand the workings of the cancer cell in enormous detail. Thirty years ago, no one could have anticipated this explosion of knowledge. Genes have led to encoded proteins, and the study of these proteins has allowed us to elucidate complex regulatory circuits transmitting signals that flux through the cancer cell and control its proliferation, differentiation, and death. Until now, relatively little of this research has had an impact on the diagnostic and therapeutic tools of the clinician. Such translation of basic research into clinical practice still lies largely ahead. But one thing is already clear: With the greatly increased understanding of the genetic mechanisms of cancer pathogenesis, many novel ways of detecting and curing tumors are now, finally, within reach.

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Oncogenes and Signal Transduction

Signaling: An Overview

Intercellular communication is critical to embryonic development, tissue differentiation, and systemic responses to wounds and infections. These complex signaling networks are in large part initiated by growth factors. Such factors can influence cell proliferation in positive or negative ways, as well as inducing a series of differentiated responses in appropriate target cells including survival, apoptosis, and differentiation. The interaction of a growth factor with its receptor by specific binding in turn activates a cascade of intracellular biochemical events ultimately responsible for the biological responses observed. Several classes of receptors are involved in transducing these extracellular signals. These include receptor tyrosine kinases, G-protein-coupled receptors, and cytokine receptors. Cytoplasmic molecules that mediate these responses have been termed “second messengers.” The transmission of these biochemical signals to the nucleus leads to the altered expression of a wide variety of genes involved in mitogenic, survival, and differentiation responses.

As knowledge has accumulated in the area of signal transduction and the complexities increase, it is becoming apparent that overlap exists in cell signaling. This functional redundancy may be seen at several levels. The simplest example would be the fact that several different extracellular signals can lead to the activation of the same pathway. Physiologically, this may serve to allow a cell to respond to a variety of different situations or stresses while conserving some of the downstream machinery. While some of the components of certain pathways may be common for two different stimuli, the ultimate physiologic response may differ greatly due to the activation of a different repertoire of nuclear response elements. Additionally, while redundancy may exist in terms of the ability of a stimulus to perturb a specific pathway, it is conceivable that the kinetics and magnitude of activation may differ, leading to distinct outcomes.

There is often redundancy between different isoforms of certain proteins or with members of particular gene families. This is illustrated by the fact that targeted disruptions of some genes fail to produce detectable phenotypes in mice, indicating that other proteins can compensate for their loss. An attractive explanation for this redundancy is that it serves as a failsafe mechanism to ensure proper functioning in the face of damaging mutations that lead

to a loss of function. Indeed, as will be discussed further, proteins within a family often have overlapping functions and may, in some situations, complement one another.

To effectively coordinate signaling cascades, nature has created a variety of molecules known as adaptor and scaffolding proteins (reviewed in Pawson and Scott [1]). These proteins play an integral role in intracellular signaling by recruiting various proteins to specific locations and by assembling networks of proteins particular to a cascade. Adaptor proteins, through protein-protein interactions via specific motifs, provide a link between molecules of a signaling cascade and proteins such as receptor tyrosine kinases (RTKs; Figure 2-1). Adaptors can be docking proteins, which provide multiple binding sites on which effector molecules can attach, thereby expanding the magnitude of responses from an activated RTK. Scaffolding proteins also exist in signaling cascades and allow the formation of multi-enzyme complexes, which are involved in a particular cascade. These are important for two reasons. The first is that the activation of an intracellular signaling cascade by a growth factor is an extremely rapid process and is not likely to occur as a result of proteins randomly floating in the intracellular milieu until they happen to come in contact with each other. Scaffolding proteins ensure the close proximity of the necessary components. The second reason is that several enzymatic components of a particular signaling cascade may be shared, although the substrates of each may differ. Thus, scaffolding proteins ensure the proper routing of signals by preventing unwanted cross-talk between pathways.

Oncogenes

Oncogenes encode proteins that possess the ability to cause cellular transformation. These genes act in a dominant fashion, either through overexpression or activating mutations. There are several criteria that define cellular transformation. These include morphologic changes, loss of contact inhibition, anchorage-independent growth, and the ability to form tumors when transplanted into nude mice. For example, under normal physiologic situations, a growth factor binding to a receptor produces a very transient activation of a certain signaling cascade allowing very tightly regulated responses such as proliferation to occur. When downstream components of these cascades are mutated in a way that causes

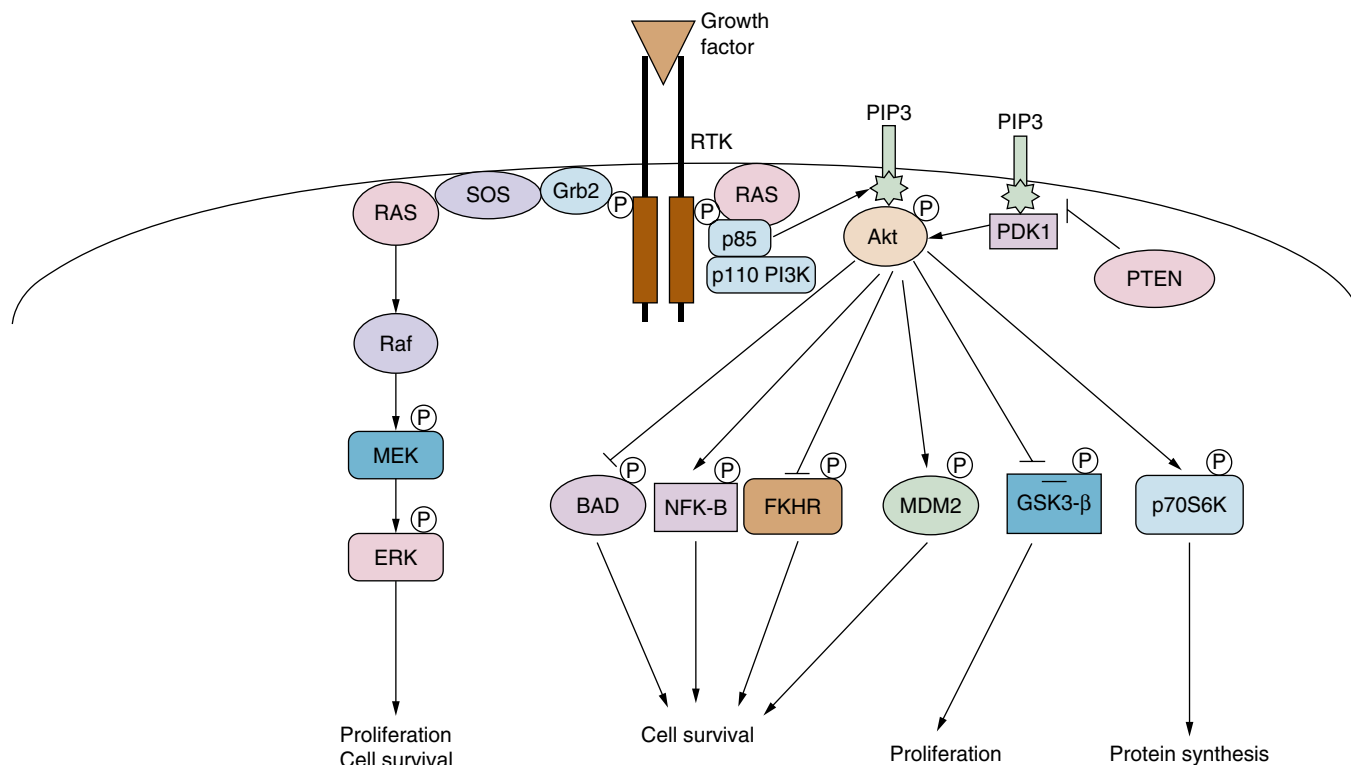


FIGURE 2-1 Receptor tyrosine kinase signaling in cancer. Scheme for growth factors signaling through receptor tyrosine kinases.

them to be constitutively active, the signal is no longer transient and regulated, but is aberrantly turned on in a continuous fashion. In addition to activating mutations, these genes can be activated by over-expression at levels much higher than in normal cells. Proto-oncogenes are commonly involved in cellular signaling, and specific examples will be discussed in the context of their roles in signal transduction.

Initially, it was believed that cellular transformation was caused solely by unregulated cell proliferation caused by activation of oncogenes. It is now known that while deregulated proliferation is most likely a necessary component for transformation, it is probably not sufficient and other changes such as modulation of cell survival functions are critical as well. In fact, as will be discussed, the function of certain oncogenes is to modulate cell survival.

In the early 1980s, approaches aimed at identifying the functions of retroviral oncogenes converged with efforts to investigate normal mitogenic signaling by growth factors. Analysis of the predicted sequences of a number of retroviral oncogene products uncovered several with similarities to the prototype *v-src* product, whose enzymatic function as a protein kinase had been identified. Unlike many protein kinases, which phosphorylated serine and/or threonine residues, the *v-src* product was a protein kinase capable of specifically phosphorylating tyrosine residues (2). Later efforts to identify oncogenes led to the discovery of the small GTP-ase Ras, which was unmasked as a transforming gene by transfection of tumor cell genomic DNA (3).

Independent efforts to purify and sequence growth factors led to the discovery that the sequence of the platelet-derived growth factor (PDGF) B chain matched the predicted product of the

transforming gene of simian sarcoma virus, designated *v-sis* (4,5). The *v-erbB* gene of avian erythroblastosis virus, which predicted a *v-src*-related protein tyrosine kinase, was then found to represent a truncated form of the epidermal growth factor (EGF) receptor (6). Independent evidence demonstrated that EGF triggering of its receptor resulted in tyrosine autophosphorylation (7). Thus, a direct link between growth factors, receptors with tyrosine kinase activity, and oncogenes was firmly established. The proliferation, differentiation, functional activity, and survival of cells can be affected by a wide array of other cytokines that signal through transmembrane receptors that lack protein tyrosine kinase activity. Because these signaling systems have also been implicated in malignant transformation, they are described in this chapter as well.

Signal Transduction by Protein Tyrosine Kinase Receptors

Receptors

Membrane-spanning RTKs contain several discrete domains, including their extracellular ligand binding, transmembrane, juxta-membrane, protein tyrosine kinase, and carboxy-terminal tail domains (8,9). Interaction of a growth factor with its receptor at the cell surface leads to a tight association, so that growth factors are capable of mediating their activities at very low concentrations. Ligand binding induces receptor dimer or oligomer formation associated with activation of the tyrosine kinase domain. Most evidence

indicates that the transmembrane domain does not directly influence signal transduction and is instead a passive anchor of the receptor to the membrane. It is important to note, however, that point mutations in the transmembrane domain of one receptor-like protein, the Neu/ErbB-2 protein, enhance its transforming properties (10). The juxtamembrane sequence that separates the transmembrane and cytoplasmic domains is not well conserved between different families of receptors. However, juxtamembrane sequences are highly similar among members of the same family, and studies indicate that this stretch plays a role in modulation of receptor function. For example, addition of PDGF to many types of cells causes a rapid decrease in high-affinity binding of EGF to its receptor. This has been shown to be a downstream effect of PDGF receptor activation in which protein kinase C (PKC), itself a serine protein kinase, is activated and, in turn, phosphorylates a site in the juxtamembrane domain of the EGF receptor (11).

The tyrosine kinase is the most conserved domain, and its integrity is absolutely required for receptor signaling. For example, mutation of a single lysine in the ATP binding site, which blocks the ability of the receptor to phosphorylate tyrosine residues, completely inactivates receptor biologic function. Yet, such kinase mutants retain the ability to bind ligand with high affinity and exhibit normal internalization and down-regulation as well (9).

The carboxy-terminal domain of the receptor is thought to play an important role in regulation of kinase activity. This region typically contains several tyrosine residues, which are auto-phosphorylated by the activated kinase. In fact, the receptor itself is often the major tyrosine phosphorylated species observed following ligand stimulation. Tyrosine phosphorylation of the carboxy-terminal can modulate kinase catalytic activity, and/or the ability of the kinase to interact with substrates. Thus, mutations that alter individual tyrosine sites or deletions of the carboxy-terminal domain have the effect of attenuating kinase function (9).

RTKs and Cancer

The constitutive expression of a growth factor and its specific receptor by the same cell may be sufficient to establish a so-called autocrine loop that contributes to tumor progression. Autocrine transforming interactions have been identified in a number of human malignancies. At least one PDGF chain and one of its receptors have been detected in a high fraction of sarcomas and in glial-derived neoplasms (12–14). Growth factors also contribute to tumor progression by a paracrine mode. For example, continuous stimulation by growth factors in paracrine as well as autocrine modes during chronic tissue damage and repair associated with cirrhosis and inflammatory bowel disease may predispose to tumors (15). Some tumor cells produce angiogenic growth factors such as the vascular endothelial growth factors (VEGFs). Such growth factors cause paracrine stimulation of endothelial cells inducing neoangiogenesis and lymphangiogenesis, which contribute to tumor progression (16).

RTKs are frequently targets of oncogenic alterations, which create a constitutively activated receptor, independent of the presence of ligand. This was initially demonstrated with retroviral oncogenes, *v-erbB* and *v-fms*, encoding activated forms of the EGF and

CSF-1 receptor, respectively (6,17). Alterations affecting a large number of RTKs have been implicated in human malignancies. One mechanism involves the amplification or overexpression of a normal receptor. Examples include the EGFR, ERBB-2, and *MET* (see reviews [18,19]). In some human tumors, deletions within the external domain of the EGFR receptor or mutations in its tyrosine kinase domain are associated with its constitutive activation (20,21). The *RET* gene is activated by rearrangement, as a somatic event, in about one third of papillary thyroid carcinomas. Germ-line mutations affecting the cysteine residues in the extracellular region are responsible for multiple endocrine neoplasia (MEN)–2A and for the familial medullary thyroid (FMTC) carcinoma syndrome. In contrast, a substitution of methionine by threonine at codon 918 in the catalytic region of the tyrosine kinase has been reported in MEN-2B (22). These mutations have been shown to up-regulate *RET* catalytic function, resulting in its genetic transmission as an oncogene. *MET* is overexpressed and/or mutationally activated in a variety of human tumors. A direct role of *MET* in hereditary papillary renal carcinoma (HPRC) has also been established (18). This hereditary disease is characterized by multiple, bilateral renal papillary tumors, in which mutations activate constitutive kinase activity and transforming properties. Somatic mutations in *MET* have also been detected in some sporadic renal papillary tumors (18). Several other receptors including the PDGF- α , TrkA, TrkC, and Alk, have been shown to be oncogenically activated in human malignancies by gene rearrangements that lead to fusion products containing the activated TK domain (23–26).

Signaling Pathways of Tyrosine Kinase Receptors

Knowledge of the cascade of biochemical events triggered by ligand stimulation of tyrosine kinase receptors has increased rapidly in recent years and provides further evidence of the importance of these signaling pathways in cancer. The PDGF system has served as the prototype for identification of the components of these systems. Certain molecules become physically associated and/or phosphorylated by the activated PDGF receptor kinase. Those identified to date include phospholipase C (PLC)- γ (27), phosphatidylinositol-3'-kinase regulatory subunit (p85) (28), Nck (29), the phosphatase SHP-2 (30), Grb2 (31), Crk (32), *ras* p21 GTPase-activating protein (GAP; 33,34), and *src* and *src*-like tyrosine kinases (35). PLC- γ is one of several PLC isoforms and is involved in the generation of two important second messengers, inositol triphosphate and diacylglycerol (36). The former causes release of stored intracellular calcium and the latter activates PKC. These second messengers appear rapidly in cells following stimulation by growth factors such as PDGF. The relative increase in their synthesis in vivo correlates reasonably well with the ability of a particular receptor kinase to induce tyrosine phosphorylation of PLC- γ (27). Moreover, tyrosine-phosphorylated PLC- γ exhibits increased catalytic activity in vitro (37). Thus, it seems very likely that receptor-induced tyrosine phosphorylation activates this enzyme. The actions of a number of tumor promoters

are thought to be mediated by PKC (36), and PKC overexpression or gene alteration has been reported to increase cell proliferation in culture.

Phosphatidylinositol-3-Kinase (PI3K) phosphorylates the inositol ring in PI in the 3' position and becomes physically associated with a number of activated tyrosine kinases (38). This protein contains an 85-kDa regulatory subunit, which is tyrosine phosphorylated, and a 110-kDa catalytic subunit. PI3-K appears to play a major role in cell survival signaling as discussed later (Figure 2-1).

Ras

Ras small GTP-binding proteins are a major point of convergence in receptor tyrosine kinase signaling and are an important component of the cellular machinery necessary to transduce extracellular signals (see review [39]). These membrane-bound intracellular signaling molecules mediate a wide variety of cellular functions including proliferation, differentiation, and survival. This family consists of 10 highly conserved proteins including H-, N-, and K-Ras, R-Ras, Rap1 (A and B), TC21, and most recently R-Ras3 (39,40). Ras proteins are synthesized in the cytosol and become associated with the inner leaflet of the plasma membrane via post-translational modifications including a form of fatty acid lipidation, isoprenylation, on Cys-186. The C-terminal CAAX box (Cys, two aliphatic amino acids, followed by any residue) is an essential motif required for Ras function as it targets the unprocessed protein for this essential modification.

Ras proteins act as molecular switches alternating from an inactive GDP-bound state to an active GTP-bound state. The paradigm for Ras activation involves the recruitment of a guanine nucleotide exchange factor (GNEF) to the membrane in response to growth factor binding and subsequent activation of a receptor tyrosine kinase (39). GNEFs promote the release of GDP from the Ras catalytic pocket, and the relative abundance of intracellular GTP as compared with GDP ensures preferential binding of GTP (Figure 2-2). The best example of a Ras GNEF is SOS (son of sevenless), which is brought to the membrane by its stable association with the adaptor protein Grb2 (Figure 2-1; 41). Grb2 contains an src-homology 2 domain (SH2), which binds to a specific motif containing phosphorylated tyrosine residues on several RTKs including the PDGFR and the EGFR. Grb2 also has two SH3 domains that mediate its binding to SOS via a carboxy-terminal proline-rich region. Alternatively, another adaptor protein, Shc, can bind to the cytoplasmic tail of the receptor through its SH2 domain resulting in its phosphorylation on tyrosine and subsequently binding Grb2 (42). The exact sequence of binding of adaptors depends on the receptor and cell type. Once SOS is translocated to the membrane, it can promote the release of GDP from Ras, allowing GTP, which is present in excess in the intracellular environment, to bind and ultimately lead to Ras activation. Although Ras is a GTPase, its intrinsic GTPase activity is actually quite inefficient and requires additional proteins known as GTPase activating proteins (GAPs) to promote GTP hydrolysis (Figure 2-2). GAPs can accelerate GTP hydrolysis by several orders of magnitude and are, thus, negative regulators of Ras functions (43). The mechanism by which GAP accelerates the GTPase reaction is complex and not

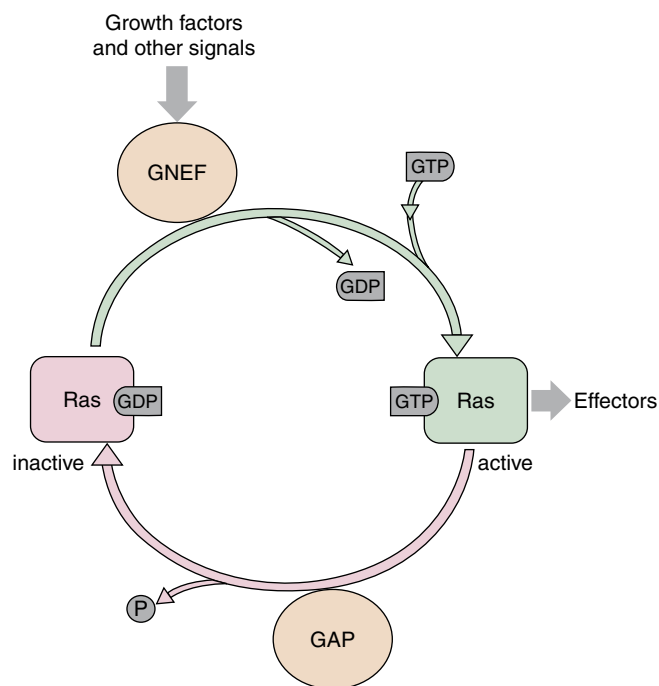


FIGURE 2-2 Activation of Ras GTPase.

completely understood. Several GAPs for Ras have been identified, including p120 GAP, NF1-GAP/neurofibromin, and GAP1^m, as well as GAPs with preferential activity on related proteins such as R-Ras (43). Of particular interest is *NF1* as it is found to be frequently inactivated by mutation in patients with the familial tumor syndrome, neurofibromatosis type I.

Ras Functions

Ras appears to have a multitude of functions that differ depending on factors such as cell type and extracellular environment. It is paradoxical that a single gene can cause cell cycle entry and DNA synthesis in one type of cell, such as fibroblasts, and terminal differentiation in others, such as PC12 (44,45). In other cell types such as myoblasts, activated Ras seems to oppose cell cycle withdrawal and differentiation into myotubes and down-regulates expression of muscle specific mRNA transcripts (46). Additionally, Ras has been demonstrated to promote cell survival in some cell types such as those of hematopoietic lineages upon cytokine withdrawal and PC12 cells and primary sympathetic neurons upon removal of nerve growth factor or other trophic factors (47,48). Although Ras mediates such important cellular processes as proliferation, survival, and differentiation, the exact contribution of H-, N-, and K-isoforms is not clear, as targeted knockouts to H- and N-Ras genes resulted in mice that did not exhibit an abnormal phenotype, while a K-Ras knockout is an embryonic lethal and exhibits liver and hematopoietic defects (49,50).

Ras and Cancer

The initial evidence for Ras involvement in cancer came from the discovery of transforming retroviruses, Harvey and Kirsten sarcoma viruses, which contained H- and K-ras cellular derived

oncogenes. The first human oncogenes were identified by transfecting genomic DNA from human tumor cell lines into NIH3T3 mouse fibroblasts and isolating the DNA fragments from the transformed foci. These were shown to be the human homologues of the viral ras genes (44). Subsequent studies have shown that Ras is oncogenically activated by mutations in over 15% of all human tumors, and in some cancers such as pancreatic carcinoma the frequency is as high as 90% (51).

Mutations in human tumors have been found at residues 12, 13, 59, and 61, with positions 12 and 61 being the most common (51). Most of these mutations decrease the intrinsic rate of GTP hydrolysis by Ras, as well as make the molecule significantly less sensitive to GAP-stimulated GTP hydrolysis. Thus, the outcome is a molecule that is predominantly GTP bound, constitutively active, and able to activate downstream pathways in the absence of growth factor stimulation. Oncogenic Ras is capable of transforming immortalized rodent fibroblasts or epithelial cells (44). Ras-transformed cells appear refractile and spindle shaped, have disorganized actin filaments, and have a decreased affinity for the substratum. They can proliferate in absence of adhesion (anchorage independence) or in the presence of low serum concentration. Such cells exhibit a loss of contact inhibition and grow to high saturation density. Of note, however, Ras alone is unable to transform primary mouse or human fibroblasts and instead causes such cells to undergo permanent growth arrest, also termed “replicative senescence,” characteristic of primary cells passed for multiple generations in culture. This senescence response appears to be dependent on the function of certain genes such as *p16^{INK4A}* and *p53*, which act as tumor-suppressor genes (52). The inactivation of these tumor-suppressor genes plays a critical role in cancer development (see Chapter 3). In fact, inactivation of *p53* or *p16^{INK4A}* allows Ras to transform these same cells, which may help to explain the selective pressure for inactivation of these tumor suppressor genes in tumors containing ras oncogenic mutations (52).

Not only is Ras itself mutated or overexpressed in cancer, but there are examples of Ras regulatory proteins that can be affected as well. The best example is NF1, a Ras GAP mentioned previously. Hereditary transmission of a defective *NF1* allele predisposes an individual to a genetic disease called neurofibromatosis type 1, or von Recklinghausen neurofibromatosis (53). Somatic mutations result in the inactivation of the second allele leading to neoplastic development. Neurofibromatosis can manifest itself with the occurrence of multiple benign neurofibromas as well as a high risk for malignancies of neural crest origin. In cells with defective NF1, cellular Ras accumulates in its GTP-bound state and thus is more active (53). Additional members of the Ras family of GTP-binding proteins can cause cellular transformation in appropriate test cells. These include R-Ras (54), TC21/R-Ras2 (55), and R-Ras3 (56).

Signaling Downstream of Ras

Ras>Raf>Map Kinase Cascade

The most well studied effector of Ras is the serine/threonine kinase Raf (Figure 2-1). Raf has been shown to bind to Ras and in many cases has been demonstrated to be indispensable for Ras func-

tions such as cellular transformation (57). In fact, activated Raf or v-Raf, a truncated form of c-raf, was initially isolated as a retroviral oncogene. There are three known mammalian Raf isoforms designated A-, B-, and C-Raf (also known as Raf-1) (see review [58]). C-Raf is ubiquitous in its tissue expression, while A-Raf and B-Raf expression are more restricted. Ras-mediated activation of Raf requires binding to two regions of this cytoplasmic kinase, both of which are located at the amino terminus.

Several phosphorylation events on both serine/threonine and tyrosine residues are believed to play a role in the full activation of Raf (58). Additionally, there are major differences in certain phosphorylation sites between B-Raf and C-Raf, indicating that regulation of activation of these two isoforms may significantly differ. There is also evidence that Raf acts as a dimer, and triggering the formation of such dimers causes an increase in its basal kinase activity.

Once activated, Raf can phosphorylate MEK (mitogen/extracellular-signal-regulated kinase kinase), a dual-specificity kinase, on Ser₂₁₈ and Ser₂₂₂ leading to its activation (see review [59]; Figure 2-1). Partial activation can be seen by phosphorylation on only one serine. There are two isoforms of MEK, designated MEK1 and -2, both of which are expressed ubiquitously with an approximate sequence identity of 80%. MEK, once activated, can in turn activate MAP kinase, also designated extracellular signal regulated kinase (ERK; 59). Activation occurs via tandem phosphorylations on both threonine and tyrosine (Thr₁₈₃-Glu-Tyr₁₈₅) with the phosphorylation on tyrosine occurring first. There are two ERK isoforms (1 and 2), ubiquitously expressed and with very similar sequences. These proteins, 44- and 42-kDa, respectively, translocate to the nucleus where they can activate a variety of proteins through phosphorylation on serine or threonine. For example, ERK can phosphorylate several of the members of the Ets family of transcription factors. Phosphorylation of Ets-1 by ERK dramatically increases *c-fos* transcription. ERK can also activate a variety of protein kinases via phosphorylation. For example, p90 RSK is a serine/threonine kinase, which has a role in protein translation and has been shown to be a substrate for ERK (60).

In addition to positive regulation of the MAP kinase pathway by phosphorylation, there are negative regulatory mechanisms that serve to attenuate activation of this cascade. A principle mode of this negative regulation is through a variety of phosphatases, a majority of which are dual specific, meaning they can dephosphorylate both serine/threonine and tyrosine residues. This is consistent with knowledge that ERK must be phosphorylated on both threonine and tyrosine to achieve maximal activation (59).

Functions of the MAP Kinase Pathway

As mentioned previously, the MAP kinase cascade mediates many Ras downstream functions (Figure 2-1). ERK activation can lead to increased DNA synthesis and cell proliferation. In fact, activated forms of Ras, Raf, and MEK induce expression of *cyclin D1*, which plays a major role in early cell cycle progression (61). Dominant-negative mutants of members of this cascade can also block this induction in response to growth factor stimulation. Of particular interest is the fact that *cyclin D1* is rearranged or amplified in human tumors and tumor cell lines, thus implicating a role for this G1 cyclin in human cancer (62).

Raf/Mek/MapK and Cancer

Davies et al. (63) identified *B-raf* mutations in around 66% of human melanoma cell lines and primary tumors. Of note, the nucleotide changes observed were not consistent with mutations typically induced by UV. Lower frequencies of analogous mutations were observed in colon carcinoma and small cell lung cancer (SCLC; 63). These mutations were further shown to oncogenically activate B-raf as determined by NIH3T3 transfection analysis. To date, mutational activation of other *Raf* genes, *Mek*, or *Mapk* has not been demonstrated in human tumors.

Other MAP Kinases

In addition to the ERKs, there are other MAP kinases belonging to distinct MAPK cascades with both different upstream activators and downstream effectors (Figure 2-3). The c-Jun

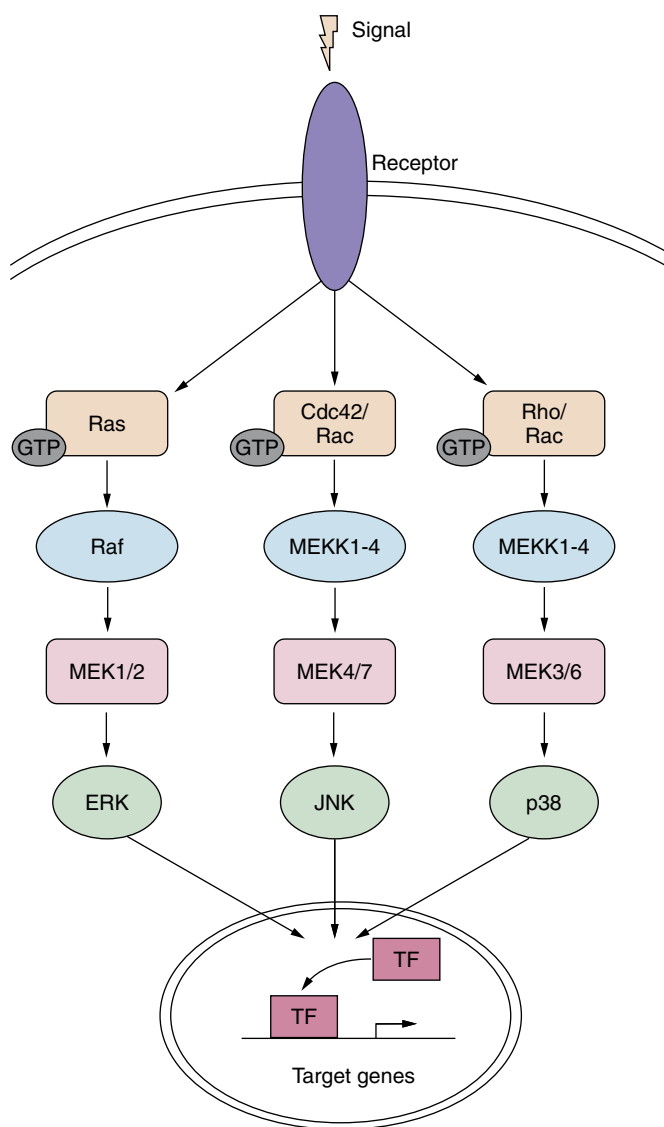


FIGURE 2-3 MAP kinase pathways in cancer. Activation of the three MAP kinase cascades ERK, JNK and p38.

N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAP kinase have been demonstrated to modulate cellular responses to a wide variety of extracellular stimuli, including mitogens, inflammatory cytokines, and UV irradiation (see review [64]). There are three JNK genes, each with several alternatively spliced transcripts. In most cell types examined, including fibroblasts, epithelial cells, and neuronal-like PC12 cells, activation of JNK/SAPK has been reported to promote programmed cell death (64). There is evidence for some redundancy among these three genes as each of the single knockouts, as well as the *JNK1/JNK3* (–/–) and *JNK2/JNK3* (–/–) double-knockouts are viable, but mice lacking both *JNK1* and *JNK2* are embryonic lethal.

In contrast to its ability to activate the MAPK/ERK cascade, H-ras only minimally perturbs JNK/SAPK. However, overexpression of constitutively activated mutants of the small G-proteins, Rac and Cdc42, leads to robust stimulation of JNK/SAPK activity. The pathways leading to JNK activation mirror those seen for ERK. Thus, a variety of MAP kinase kinases (MKK) can phosphorylate the various JNK isoforms (59,64).

As with the ERKs, JNK activation results in phosphorylation of certain transcription factors and increases their transcriptional activity at promoters containing response elements for these factors (64). Some of the transcription factors activated by ERK or JNK were initially discovered as retroviral oncogenes in mice and chickens respectively. The FBJ and FBR murine viruses contain the *fos* sequence under the viral LTR promoter and exhibit changes in regulatory phosphorylation sites that make them more active than the proto-oncogene (65) and *c-jun* was identified as an avian retrovirus (66). Overexpression of *c-fos* can cause transformation of cells as well (67). *c-fos* and *c-jun* together comprise the AP-1 transcription factor. This heterodimer, in response to UV irradiation, environmental stresses, and PKC activation binds to AP-1 target sequences such as 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive elements (68).

C-Myc

The myc family includes four transcription factors, *c-myc*, N-myc, L-myc, and S-myc, involved in the control of cell growth, differentiation, and apoptosis (69). Myc proteins form heterodimers with another transcription factor, Max, through a basic-region/helix-loop-helix/leucine-zipper domain and bind a specific DNA consensus sequence called E-box to activate the transcription of target genes. In the absence of myc, Max forms a complex with Mad/Mnt proteins and acts as a repressor of the transcription. The large number of genes regulated by myc, which includes *p21^{CIP1}*, *cyclin D1/D2*, and *E2F2*, make it difficult to identify the crucial targets for its functions (a recent estimate proposed that myc can bind to ~25,000 sites in the human genome; 69). Enhanced expression of myc proteins is associated with various types of malignancies. However, the deregulated expression of these proteins is not sufficient to induce cell transformation, implying that additional genetic events are required. One such event is the activation of the Ras pathway, which affects myc factors at different levels, including post-translational stabilization and inhibition of the antagonizing transcription factors FOXO (69). It has been shown that *c-myc*, the cellular form of the *v-myc* viral oncogene, is induced

by growth factors in quiescent fibroblasts and can cooperate with ras to cause transformation of these cells (44). *c-myc* is altered in a large fraction of human cancers, although its role in the progression to malignancy is still not completely understood. In lymphoid cancers, *c-myc* is often found in translocations adjacent to a strong promoter such as that of the immunoglobulin genes. In other cancers such as breast and lung carcinoma, the genomic locus encoding *c-myc* is amplified. Gene amplification is also the mechanism responsible for the increased expression of *N-myc* commonly observed in certain cancers, including retinoblastoma, glioblastoma, and medulloblastoma. Of note, *N-myc* overexpression in neuroblastoma strongly correlates with an advanced clinical stage and it is taken into consideration for the assessment of the treatment of these malignancies (69,70).

Oncogenes and Survival Signaling

The regulation of cell survival and cell death is of extreme importance in both the development of an organism as well as in the physiologic functions of the adult. During development of a multicellular organism, certain cells are eliminated by a process known as apoptosis or programmed cell death and others permitted to survive. The deregulation of these processes can lead to a variety of malformations resulting in deformities or, in extreme cases, incapability with life. In adulthood, regulation of cell survival is equally important for proper homeostasis. Damaged cells must be removed, and terminally differentiated cells must be sustained. A failure for this to occur may result in the accumulation of mutations leading to cancer. Pro-apoptotic and anti-apoptotic proteins regulate these processes, and many of the oncogenes already discussed modulate cell survival in a positive fashion. Thus, oncogenes can influence proliferation, cell survival, or both, contributing to cellular transformation in a cooperative fashion.

The Bcl-2 Family

The Bcl-2 family of proteins consists of more than 15 members that can be subdivided into three classes on the basis of functions and the number of Bcl-2 homology (BH) domains present (71,72). The anti-apoptotic members include Bcl-2 and Bcl-XL, which contain four BH domains (BH1 to BH4). The pro-apoptotic members such as Bax and Bak have three BH domains (BH1 to BH3), and the “BH3 only” pro-apoptotic proteins, such as Bid and Bim, contain only the BH3 domain. Proteins in all three classes have the ability to form either homo- or heterodimers with one another and play distinct roles in regulating mitochondrial membrane permeabilization (MMP; Figure 2-4; 71,72).

The involvement of Bcl-2 and cancer has been firmly established. Not only was the gene cloned as a translocation from a lymphoid tumor as stated previously, but mice expressing a Bcl-2-immunoglobulin “mini-gene” that mimicked the translocation seen in human cancers, showed follicular hyperplasia that progressed to lymphoma. The Bcl-2 genomic locus is translocated in several tumor types including follicular lymphomas and chronic lymphocytic

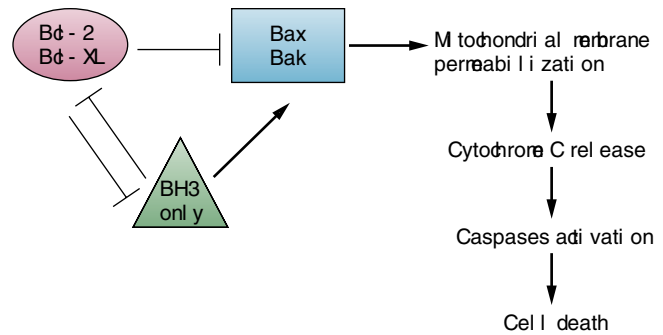


FIGURE 2-4 Bcl-2 family members interactions regulate cell death.

leukemia. Moreover, other oncogenes such as Ras and Myb induce Bcl-2 expression. Overexpression of anti-apoptotic family members as well as down-regulation or inactivation of pro-apoptotic proteins has been observed in several human cancers (71,72).

PI3K-Dependent Pathways

PI3K is a lipid kinase that catalyzes the transfer of the γ -phosphate from ATP to the D3 position of phosphoinositide (PtdIns) generating PtdIns3P, PtdIns(3,4)P₂, and PtdIns(3,4,5)P₃ (Figure 2-1; 38). These lipids can act in a variety of cascades. PI3K activation has been demonstrated to play an important role in cell survival signaling in a number of cell types. There are three classes of PI3Ks, which exhibit variability with respect to their method of activation or their preferred lipid substrate.

The prototypical class 1 PI3K consists of two subunits encoded by two distinct loci; a regulatory and a catalytic subunit (38). The regulatory subunit is a 50- to 85-kDa protein that is tightly associated with the p110 catalytic subunit. The classical mode of PI3K activation involves its binding to the phosphorylated tyrosine residues of receptor tyrosine kinases via the two SH2 domains of p85. This results in a conformational change, which is believed to facilitate activation of the p110 catalytic activity (38). Additionally, it has been demonstrated that PI3K can be activated independently of receptor binding by the small G-protein Ras. The activation of PI3K by Ras is still somewhat controversial as there is also evidence that Ras is downstream of PI3K (Figure 2-1; 73). Additionally, the γ isoform of PI3K is activated by heterotrimeric G-proteins (74). Thus it is clear that PI3K can be activated in response to a wide variety of upstream signals.

There are several known downstream effectors of PI3K. These include Rac, p70^{s6k}, certain isoforms of PKC and most relevant to the discussion of cell survival, Akt/PKB (38,75). Akt has been shown to be responsible for PI3K-dependent cell survival signaling and is the cellular homologue of the viral oncogene *v-Akt*. The three human homologues identified encode 57-kDa serine/threonine kinases that contain an N-terminal PH domain, which binds to the activated PtdIns products of PI3K. These lipids are believed to mediate the localization of this cytoplasmic protein to the plasma membrane. In addition, phosphorylation of Akt on two residues, a serine and a threonine, is required for full

activation. These events are catalyzed by two different kinases, one of which, PDK1 (PtdIns(3,4,5)P₃-dependent kinase) specifically phosphorylates Thr³⁰⁸ and the other, PDK2, phosphorylates Ser⁴⁷³. The identity of PDK2 is still controversial, although studies have suggested that a complex of the mTOR (mammalian target of rapamycin) kinase and the adaptor rictor may be responsible for this critical phosphorylation of Akt (38,75–77).

Several reports have shown that Akt promotes survival and prevents apoptosis in various cell types. Akt phosphorylates the pro-apoptotic bcl-2 family member, BAD, both *in vitro* and *in vivo* on Ser¹³⁶. When BAD is phosphorylated, it gains affinity for the cytosolic protein, 14-3-3, and forms a complex with this protein. Phosphorylated BAD cannot heterodimerize with the anti-apoptotic bcl-2 family member, Bcl-X_L, permitting free Bcl-X_L to protect the cell from apoptosis (Figure 2-1; 75).

The striking anti-apoptotic effect of both PI3K and its downstream effector, Akt, as well as the fact that they were initially found as transforming viral oncogenes, suggested that these two genes might also be involved in human cancer. Indeed, a myristoylated constitutively active PI3K can cause cellular transformation in chicken embryo fibroblasts (78). The genomic locus encoding the p110 α catalytic subunit of PI3K was found to be amplified in a high percentage of ovarian tumors and ovarian tumor cell lines, and evidence indicates that activating mutations are present frequently in a variety of tumors (75). There is also evidence for Akt involvement in human malignancies. *Akt1* was found to be amplified 20-fold in a primary gastric adenocarcinoma. Additional studies have shown genomic amplification and overexpression of *Akt2* in several pancreatic and ovarian carcinoma cell lines as well as amplification in some of ovarian and breast carcinomas examined (75). Of particular note is the fact that overexpression of *Akt2* occurs more frequently in undifferentiated and, thus, more aggressive tumors.

Further evidence of the involvement of the PI3K/Akt pathway in cancer stems from the discovery of the PTEN/MMAC tumor suppressor, a gene mutated in a high fraction of glial and endometrial tumors as well as in melanoma, prostate, renal, and small cell lung carcinomas (79). Germ-line mutations at the PTEN locus cause inherited cancer syndromes such as Cowden disease, Lhermitte-Duclos disease, and Bannayan-Zonana syndrome. PTEN dephosphorylates the 3 position of phosphatidylinositol *in vitro* and *in vivo*. Thus, PTEN directly opposes PI3K activity by dephosphorylating its activated lipid products (Figure 2-1; 75,79).

Cytokine Receptor Signaling

A large number of cytokines, hormones, and growth factors have been shown to activate a class of receptors that lack significant sequence similarity to the RTKs, and are grouped under the definitions of cytokine receptors (80). They share a common structural motif in their extracellular domains including conserved cysteine residues and lack intrinsic enzymatic activity. The cytokine receptors either homodimerize upon ligand binding (receptors for

growth hormone, prolactin, erythropoietin, and thrombopoietin) or are composed of two distinct subunits that heterodimerize in response to ligand interaction. This latter group of receptors is composed of a ligand-specific chain and a common chain shared by different cytokines. This includes the receptors for interleukin-6 (IL-6), IL-11, oncostatin-M, LIF, cardiotrophin-1 and ciliary neurotrophic factors, all sharing a common chain called gp130, the receptors for IL-3, IL-5, and GM-CSF that share the common β chain and the receptors for IL-2, IL-4, IL-7, IL-9, IL15 that share the common γ common chain.

The Janus kinases (JAKs) originally identified as signaling molecules in the interferon pathway are essential transducers of the signal originating from cytokine receptors (81,82). Four mammalian JAKs have been identified, Jak-1, Jak-2, Jak-3, and Tyk-2. These kinases are associated with the receptors and become phosphorylated upon ligand binding; the activated JAKs cause phosphorylation of the receptor and of molecules containing either a phosphotyrosine binding domain (PTB) or a SH2 domain (SH2). These molecules comprise the signal transducers and activators of transcription (STATs).

Seven mammalian STATs have been identified (Stat-1, Stat-2, Stat-3, Stat-4, Stat5a, Stat5b, and Stat-6), and differential splicing increases the number of these molecules. In addition to an SH2 domain, they contain a DNA-binding domain and several protein–protein interaction domains. After becoming phosphorylated on tyrosine, STATs form homo- or heterodimers through their SH2 domain and translocate to the nucleus where they activate target genes (Figure 2-5; 81,82). In addition to the cytokine receptors, the Jak/STAT pathway has been shown to transduce signals from a number of tyrosine kinase receptors including those for EGF, PDGF, and CSF-1 (82).

Several components of the cytokine receptor signaling pathways have been implicated in uncontrolled cell proliferation and cancer. In experimental models, oncogenic activation of the Epo receptor can occur via receptor mutations, which constitutively up-regulates its functional activity and cause transformation of appropriate hematopoietic target cells (83). Another acute transforming retrovirus, the myeloproliferative virus (MPLV), contains an oncogene called *v-mpl*, which is a truncated version of a member of the hematopoietin receptor family *c-mpl* whose ligand has been identified as the thrombopoietin (TPO). The *c-mpl* receptor can also be activated *in vitro* by mutations (84) and its ectopic expression in mice induces a lethal myeloproliferative disease (85). The first evidence of involvement of the JAKs/STATs in naturally occurring cancer was the finding of an activating mutation in the *Drosophila* Hop kinase, a member of the JAK family, that caused a leukemia-like phenotype (86). Constitutive activation of the JAKs/STATs has also been observed in a number of cell lines, including HTLV-1-infected cell lines and cell lines transformed by viral oncogenes like *v-src* and *v-Eyk* and herpes virus Saimiri-infected cells. Constitutive activation of the JAK/STAT pathway has been found in acute and chronic myelocytic leukemia and in chronic lymphocytic leukemia (87). Stronger evidence implicating the JAKs in a human cancer came from the identification of a chromosomal translocation in a human leukemia resulting in the constitutively activated fusion protein, TEL-JAK2 (88). Persistently

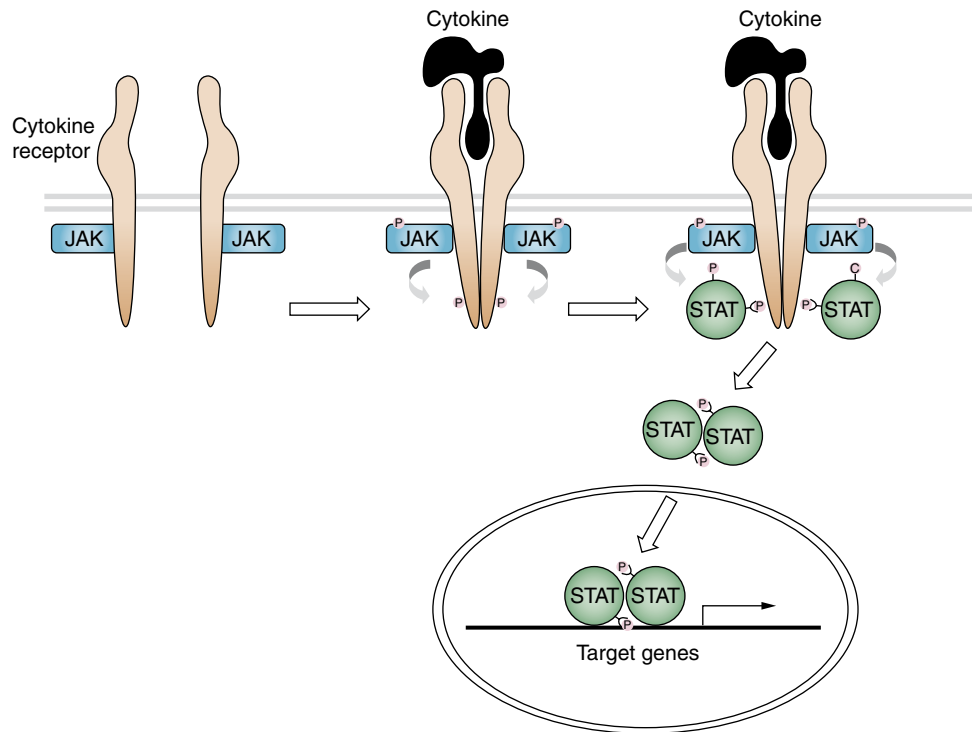


FIGURE 2-5 CYTOKINE RECEPTOR SIGNALING. The binding of cytokines to their receptors activates the JAK/STAT pathway through a series of phosphorylations.

activated STATs in the absence of evidence of mutations in the STAT genes themselves have been reported in a variety of human cancers (87).

Neurotransmitters

The transmission of signals generated by the reception of chemical and physical stimuli from the external and internal environments is mediated by a large variety of small molecules known as neurotransmitters. These molecules include acetylcholine; amino acid derivatives such as epinephrine, norepinephrine, serotonin, and dopamine; and peptides such as the angiotensins, β -endorphin, enkephalins, and somatostatin. These ligands can trigger two types of receptors: ion-channel-linked receptors or receptors with seven membrane-spanning domains, which interact with heterotrimeric G-proteins composed of α , β , and γ subunits. After binding to their specific ligand, the G-protein-coupled receptors (GPCRs) undergo a conformational change, which results in a switch from the inactive GDP-bound G_α to an active GTP-bound state and the dissociation of the $G_{\beta\gamma}$ subunits. Different subfamilies of G_α proteins exist that activate various signaling pathways. For example, G_{α_s} and G_{α_i} , respectively stimulate or inhibit adenylyl cyclase (AC), provoking an increase (or a decrease) of cyclic AMP (cAMP) levels, which can then activate the protein kinase A (PKA) (Figure 2-6). The members of another G_α subfamily, G_{α_q} , activate PLC β , which catalyzes the cleavage of phosphatidylinositol biphosphate (PIP $_2$)

into diacylglycerol (DAG) and inositol triphosphate (IP $_3$). DAG then stimulates protein kinase C (PKC), while IP $_3$ mobilizes the intracellular store of calcium (Figure 2-6). The $G_{\beta\gamma}$ subunits are also implicated in the signaling cascade by regulating the activity of different effectors, such as phospholipases, ion channels, and various kinases. Of note, GPCR activation can impinge on other transduction pathways, including Rho and Ras GTPases or MAP kinases, while the mechanisms involved are not completely elucidated (for review [89]).

The ability of GPCRs to activate various transduction pathways that regulate cell differentiation and proliferation strongly suggests a potential role of these receptors in tumorigenesis. Indeed, activating mutations of the thyroid-stimulating hormone receptor commonly occur in thyroid adenomas and carcinomas, and germ-line mutations cause familial nonautoimmune hyperthyroidism (89). Another example is illustrated by studies on two distinct groups within a subset of growth hormone-secreting human pituitary tumors (90). In one group, G_{α_s} was found to be constitutively active, resulting in elevated adenylyl cyclase activity and growth hormone levels. This activation was due to point mutations in either a site at which cholera toxin inactivates G_{α_s} [Arg 201 $\rightarrow$ Cys/His] or at a residue equivalent to a GTPase-inhibiting mutation that causes malignant activation of Ras p21 [Gln 227 $\rightarrow$ Arg]. Because both mutations have the effect of destroying GTPase activity, G_{α_s} [designated *gsp*] becomes constitutively activated in a manner analogous to the oncogenic activity of Ras p21. The two mutations are located in regions that are highly conserved among G_α proteins isolated from diverse eukaryotic species, and

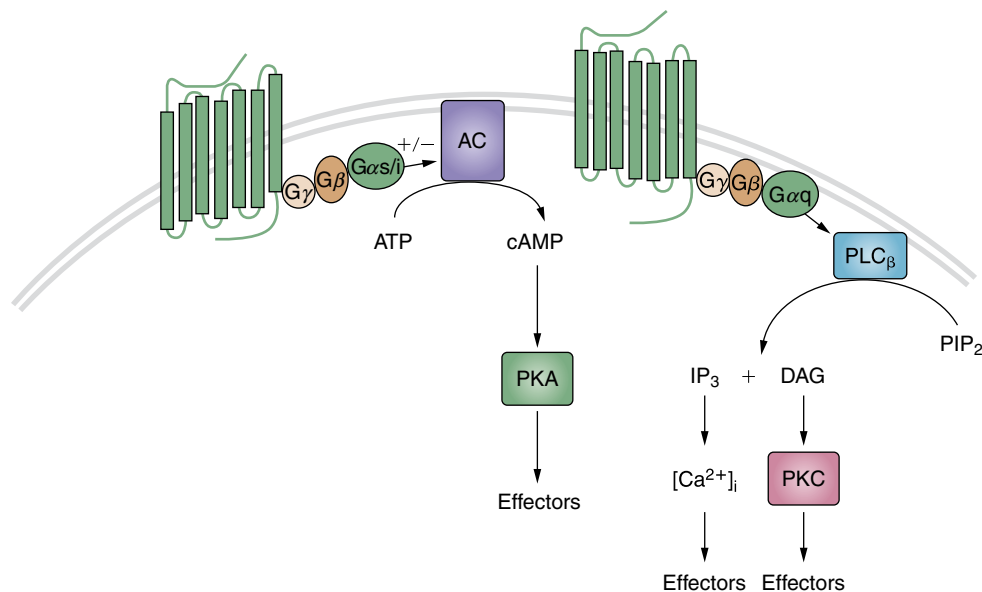


FIGURE 2-6 G-PROTEIN-COUPLED RECEPTORS (GPCRs). Diagram showing cyclic adenosine monophosphate (cAMP) and phospholipase C- β (PLC β) transduction pathways activated by GPCRs.

activating mutations have been identified in some human adrenal, pituitary, and other endocrine tumors (91). Although mutations in GPCRs and G proteins have been identified in some tumors, the most common mechanisms of GPCRs activation in cancer cells are receptor overexpression and autocrine stimulation (89), as it has been shown, for example, for the gastrin-releasing peptide (92,93), angiotensin II (94), and cholecystokinin (95) in pancreas and prostate cancers.

The effects of some neurotransmitter receptors in cancer cells and their specific expression in tumors arising from the endocrine system make this signaling a promising target for cancer diagnosis and therapy (96). Of note, other families of ligands triggering GPCRs play important roles in carcinogenesis and tumor progression. This is the case, for example, of the prostaglandins, which mediate chronic inflammation increasing the risk of tumors, and the chemokines, crucially involved in cancer metastasis (89).

Wnt Signaling

Wnts comprise a highly conserved multimember ligand family, which play important roles in a variety of developmental processes, including patterning and cell fate determination (97,98). Recent evidence indicates that in certain adult tissues, Wnts play important roles in stem/progenitor cell proliferation and differentiation (99–101). Wnt binds to two coreceptors, the seven-transmembrane Frizzled and single-membrane-spanning LRP5/6. Coreceptor activation leads to recruitment of axin and dishevel proteins to the cell membrane and to inhibition of the serine threonine kinase GSK-3. GSK-3 normally phosphorylates β -catenin as part of a multiprotein complex involving GSK-3, APC, and Axin (102). Phosphorylation targets β -catenin degradation through the ubiqui-

uitation pathway. Wnt-induced inhibition of GSK3 results in the inhibition of β -catenin degradation and its accumulation in the cytoplasm in an uncomplexed form. The latter is then translocated to the nucleus and through interaction with the TCF/LEF transcription factors activates transcription (Figure 2-7; 102). Target genes of the β -catenin-TCF/LEF complex include the proto-oncogene *c-myc* and *cyclin D1*.

The prototype *Wnt* gene was originally identified as a cellular gene activated by integration of the mouse mammary tumor virus (98). Later studies indicated that targeted expression of certain Wnts in transgenic mice caused mammary gland hyperplasia, and several *Wnt* genes exhibit the ability to transform different epithelial (103) and fibroblast murine cell lines (104). Evidence indicates that Wnt signaling is constitutively activated in some breast and ovarian tumors by an autocrine mechanism (105). More commonly, specific downstream components of the Wnt pathway have been implicated in human cancers. Genetic alterations of β -catenin have been identified in human tumors and cancer cell lines, including colon cancer, melanomas, and hepatocellular carcinomas (102). These mutations affect the sites of phosphorylation of β -catenin by GSK-3 and result in the inhibition of its degradation leading to the stabilization of the protein in the cytosolic and/or nuclear compartments.

The APC tumor suppressor gene product, which is required for β -catenin phosphorylation and degradation, also regulates the amount of cytosolic β -catenin. Inactivation of the APC gene leading to increased cytosolic β -catenin is found in 80% of colon cancers, and its inactivation occurs at an early stage in tumor progression. Germ-line mutations in the APC gene are also responsible for familial adenomatous polyposis (FAP), a dominantly inherited syndrome characterized by the formation of hundreds of colorectal adenomas, some of which inevitably progress to colorectal cancer. APC mutations cause inhibition of β -catenin degradation, resulting in activation of β -catenin signaling. The major initiating event

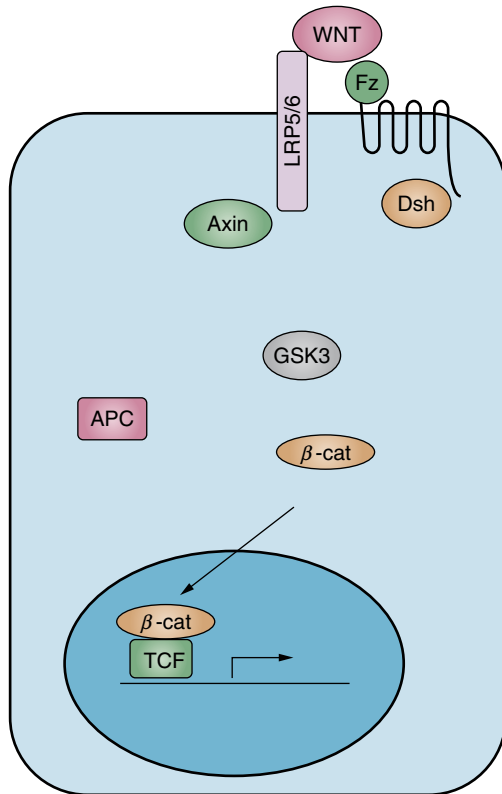


FIGURE 2-7 Diagram showing the major known components of the Wnt signaling in cancer. Simplified scheme of canonical Wnt signaling.

in the remaining 15% of colon cancer involves mutations in the *β-catenin* gene, which occurs only in those cancer cells with intact APC, implying that activation of this signaling pathway occurs in almost all colon cancers.¹⁰²

Hedgehog/Patched Signaling

The hedgehog/patched signaling pathway was first identified in *Drosophila* where it plays an important role in a number of developmental processes, including cell fate determination and patterning (106,107). Although the major core components of the pathway are conserved among species, many differences exist between the fly and vertebrate hedgehog signaling. In vertebrates, in the absence of the hedgehog ligands, sonic, Indian and desert hedgehog, their receptor patched prevents, through an unknown mechanism, the accumulation of the seven-transmembrane domain protein, Smoothened, at the cell-surface. Under such conditions, several proteins, including suppressor of fused, iguana, and costal-2, participate in the activation of the transcription factor, Gli1, and the cleavage of Gli3, which results in a repressor form of Gli that inhibits the transcription of hedgehog target genes. The third member of the Gli family, Gli2, is also cleaved but it acts as a weaker repressor (Figure 2-8A). Hedgehog binding to patched relieves the inhibition of smoothened, which can then translocate to the plasma membrane. The active smoothened triggers an as-yet-undefined cascade of events that culminates in the inhibition of Gli2/3 repressors and the accumulation of activated forms of Gli1/2, resulting in the expression of the target genes (Figure 2-8B; 108–110).

Several lines of evidence suggest an important role of the hedgehog pathway in cancer. Mutations in the human homologue of the *patched* gene have been identified as responsible for the hereditary nevoid basal cell carcinoma syndrome (NBCCS; 111), and mutations have also been found in sporadic basal cell carcinomas (BCCs), and medulloblastomas. Loss of patched would result in the constitutive activation of smoothened and up-regulation of this signaling pathway. Other studies have identified activating

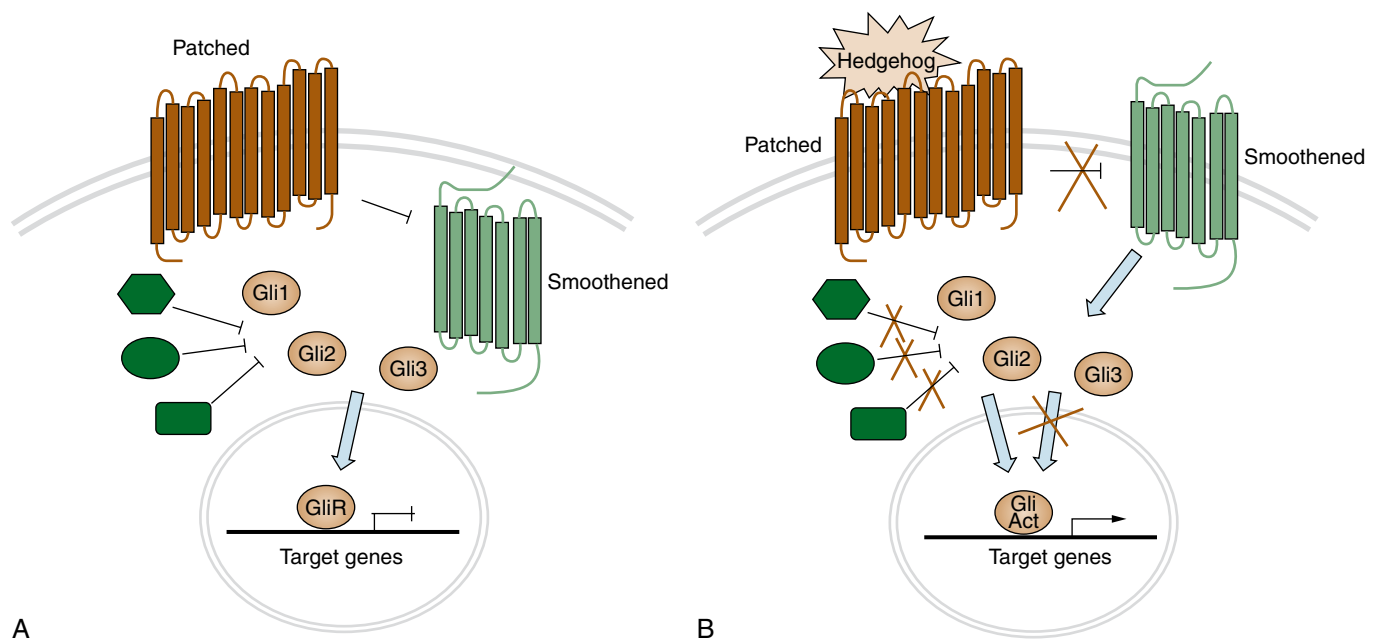


FIGURE 2-8 ACTIVATION OF THE HEDGEHOG SIGNALING. Hedgehog pathway in vertebrates, in the absence (A) or in the presence (B) of hedgehog ligands.

missense mutations in *smoothened* in sporadic basal cell carcinomas (112), further supporting the involvement of this signaling pathway in human cancer. Studies have implicated autocrine hedgehog activation as playing an important role in essentially 100% of prostate, upper gastrointestinal tract, and small cell lung carcinomas (106,107,110).

Implications for Cancer Therapy

The study of signal transduction is crucial to the understanding of the normal cellular processes that govern cellular functioning. While our knowledge of these intricate events is increasing rapidly, the complexities appear to be growing even more rapidly. What was once believed to be rather simple and linear pathways has now become multidimensional. Signaling pathways converge, diverge, and cross-talk so frequently that it is becoming difficult to discuss them as individual pathways. Issues such as cell type specificity, where signaling pathways differ both in how they are activated as well as in the ultimate outcome, add to the complexities. The oncogenes that have been discussed are normally key players in signaling pathways as illustrated by evidence that constitutive activation of molecules, ranging from receptors to nuclear transcription factors, can cause cellular transformation and/or increased cell survival, and are commonly found to be activated in human cancers.

Since a number of the signaling pathways involved in cellular transformation by oncogenes have been elucidated, concerted efforts have been made to develop treatment strategies that target these specific signaling molecules or their downstream effectors. This type of therapy has great potential as it relies on blocking specific molecules rather than the traditional chemotherapy or radiation therapy. Tremendous strides have been made in developing therapies that target oncogene products expressed at the cell surface such as *erbB*/*HER2*/*neu* or the EGF receptor using humanized monoclonal antibodies (mABs) that inhibit ligand/receptor interactions or cause receptor down-regulation and may also induce host-mediated immune responses (113–117). These mABs have been particularly effective when used in combination with traditional agents, and have in some cases been approved for first-line therapy of tumors that exhibit the specific oncogenic alterations as in the case of *erbB2* amplification in some breast cancers (Table 2-1; 115,118).

Table 2-1 Targeted Therapeutics Directed against Oncogene Products

Target	Cancer Drug Monoclonal Antibody	Disease
ErbB2	Trastuzumab (Herceptin)	Breast cancer
EGFR	Cetuximab (Erbix)	Colorectal cancer
VEGF	Bevacizumab (Avastin)	Colorectal cancer, NSCLC
Small Molecule		
Abl, PDGFR, c-Kit	Imatinib (Gleevec)	CML, GIST
EGFR	Gefitinib (Iressa)	NSCLC
EGFR	Erlotinib (Tarceva)	NSCLC
VEGFR, PDGFR, FLT3, c-Kit, Raf, Ret	Sorafenib (Nexavar)	RCC
VEGFR, PDGFR, FLT3, c-Kit	Sunitinib (Sutent)	GIST, RCC

CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumor; NSCLC, non-small cell carcinoma; RCC, renal cell carcinoma.
Drugs included in this table have been approved by the Food and Drug Administration (FDA).

Small-molecule inhibitors that inhibit the tyrosine kinase activity of RTKs have also moved successfully to the clinic for use in combination with traditional agents (Table 2-1; 119). Another treatment modality that derives from increased understanding of growth factor signaling that occurs in the microenvironment of a tumor is the application of an mAB directed against the angiogenic growth factor, VEGF, which has been approved as a novel therapy in combination with other modalities. Whether this mAB acts by inhibiting tumor angiogenesis or by normalizing such vessels and actually improving access of traditional agents to the tumor is being evaluated but there is little question that this approach can have therapeutic effects (Table 2-1; 116). Other approaches to target the tumor microenvironment are in different stages of clinical development (116,119,120). Pharmaceutical companies are now developing inhibitors to several signaling molecules including Ras, Raf, MEK, and PI3K. In summary, increased knowledge of oncogene signaling pathways has already led to novel therapeutics, which are in the clinical setting, and there is great promise that the number of rationally based therapies using such molecules as targets will continue to grow.

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Tumor Suppressor Genes

Over the past 40 years a large number of diverse research approaches have elucidated the origins of cancer development in humans and animals (see Chapter 1). Mutations in three classes of genes can help to promote the formation of cancers in humans; these genes are the oncogenes, the tumor suppressor genes, and genes involved in DNA damage repair processes. The oncogenes suffer mutations that deregulate or activate their protein products so that they function at higher levels, activities, or at inappropriate times and places in a cell. Mutations in oncogenes act in a dominant fashion (act as gain-of-function mutations) so that only one of the two alleles in a cell is commonly affected; these mutations can be gene amplifications, promoter mutations that increase the levels of a protein, translocations that produce fused and altered proteins, or missense point mutations at selected places in a gene and its protein that activate an activity or produce a protein that can not be properly regulated or degraded. Growth factor receptors, protein kinases, G-protein–signaling molecules, and transcription factors in selected signal transduction pathways are common targets for oncogene mutations (1).

Tumor suppressor genes commonly contribute to the fidelity of the cell cycle replication process. They may act as negative regulators of oncogenes, cell cycle check points, or gene products that supply the appropriate nutrients or components to complete a faithful cell cycle division in the absence of stress. Mutations in tumor suppressor genes are loss-of-function mutations and so occur in both alleles of a gene (the mutations act in a recessive fashion). Deletions, nonsense mutations, frame-shift mutations, insertions, or missense mutations that inactivate functional activity of a protein are all observed in tumor suppressor genes. Some tumor suppressor genes have a haploinsufficient phenotype. In animals or humans with one mutant allele and one wild-type allele of a tumor suppressor gene, a suboptimal level of the gene product results in a lower level of function and a loss of fidelity. The *p53* tumor suppressor gene in the heterozygous condition has a lower level of apoptosis in lymphocytes exposed to stress in both mice and humans (Li-Fraumeni syndrome) when compared to two wild-type copies of that gene (2). It is rare (the square of the independent probabilities) that the same gene in the same cell of an organism is mutated two independent times. Rather tumor suppressor genes accumulate two mutations in the same gene by a process termed “reduction to homozygosity,” which is mediated by either gene conversion (via replication or recombination) or loss of one chromosome (with the wild-type

allele) and duplication of the chromosome with a mutant allele on it. The net result of these events is two mutant alleles of a tumor suppressor gene (3).

The third class of genes that harbor mutations that contribute to cancers are genes whose products contribute to DNA repair processes. DNA damage is common and many types of DNA damage are brought about by different types or forms of mutagens or carcinogens in the environment. Evolutionary processes have endowed all organisms with multiple DNA repair systems to repair single- or double-strand DNA breaks, chemical modifications of DNA bases or sugars, photo-reactive products from radiation, and so forth. When genes in these pathways fail to function because of mutations in these genes, then DNA repair processes fail. The act of replicating damaged DNA or chemically altered DNA raises the mutation rate many fold and this enhanced mutation rate contributes to the accumulation of mutations in oncogenes and tumor suppressor genes. Mutations in tumor suppressor genes and genes involved in DNA repair, but not oncogene mutations, have been observed in the germ line of humans and animals and therefore contribute to the inherited basis of cancers (3). Mutations in both oncogenes and tumor suppressor genes are observed as somatic mutations in cancerous tissues.

Most cancers appear to contain a number of diverse oncogene and tumor suppressor gene mutations. In addition, some cancers have a high level of genomic instability or rates of mutation, perhaps due to mutations in DNA repair systems or cell cycle check points or a loss of homeostatic mechanisms in a cell. It is thought that the properties of a cancer (aggressive, invasive, indolent, fast or slow growth rate) may come from the combinations of specific oncogenes and tumor suppressor genes that are mutated in a cell. Because there are perhaps hundreds of potential oncogenes and 30 to 50 tumor suppressor genes and hundreds of genes involved in DNA repair, the combinatorics of the origins and mutational evolution of cancers is large and this helps to explain the observed heterogeneity of cancers of even the same cell or tissue type or even in the same family. What is less well understood is that not all of the oncogenes identified in animal cancers or viruses that cause cancers in animals are observed to harbor mutations in human cancers. Cancers of a specific cell or tissue type in humans only seem to use a selected subset of possible oncogenes or tumor suppressor genes. Indeed when a tumor suppressor gene mutation is transmitted via the germ line it may well give rise to cancers of

different cell or tissue types than when it is found as a somatic mutation. Even selected DNA repair defects, which are thought to act in all tissues of the body, give rise to cancers in only a subset of tissues, even though the defect is thought not to be tissue specific. It appears that there is tissue specificity in the selection of mutations so that only a subset of genes are observed to be mutated in a specific type of cancer. This is only partially understood at this time. In addition, it has been a common observation that the multiple mutations in oncogenes and tumor suppressor genes in a cancer occur in gene products that function in different signal transduction pathways that perform different functions. Therefore, cancers have a collection of mutations that inactivate or activate four or five different signal transduction pathways. Many of these pathways have multiple oncogenes and tumor suppressor genes and which genes are selectively mutated can often depend on the cell or tissue type of the cancer. It is becoming clear that these signal transduction pathways and the oncogenes and tumor suppressor genes that populate these pathways may function differently in different cell or tissue types, and this can give rise to the remarkable tissue specificity that is observed. Clearly there are a number of variables in the combinatorial pattern of observed mutations in a cancer that we need to understand.

The Concept of Tumor Suppressor Genes

The first experimental indication of genes that had the properties of tumor suppressor genes (prevent cancers from occurring) came from somatic cell genetics. Harris and Klein (4) carried out a set of experiments in which normal cells in culture were fused with tumorigenic cancer cells. An analysis of the cloned hybrids produced showed that they had many of the properties of normal cells in culture, and even when injected into an animal, they commonly failed to produce a tumor while the malignant parent alone did produce tumors. This suggested that the normal cell contributed a tumor suppressor that overrode the cancer phenotype. This interpretation was tempered by the fact that the somatic hybrid cell had at least twice the number of chromosomes found in a normal cell and gene dosage was clearly abnormal. Occasionally these hybrid cells did form reproducible tumors in animals. When the chromosome content of these tumors was examined, several of the chromosomes from the normal parent were lost and it was sometimes possible to observe that a common chromosome was always lost in the tumorigenic hybrid. This suggested that a specific chromosome carried a tumor suppressor gene in the normal cell. When two different cancerous cells were fused to form a hybrid, under some circumstances the offspring was normal. This was interpreted as two cells with different tumor suppressor genes inactive so that the hybrid cell complemented the defects of the inactive recessive tumor suppressor genes. While not a perfect experimental system, the results of this analysis using somatic cell genetics suggested the existence of tumor suppressor genes.

About this same time Knudson and his colleagues (5) were trying to explain how a single type of childhood tumor, retinoblastoma (Rb), could present in the clinic in two very distinct ways. Some children developed retinoblastoma at a young age: from

birth to 2 years old. These children almost always had bilateral tumors (in both eyes) and often had two to four tumors per eye. Other children first developed these tumors from 3 to 7 years of age, and these patients had only one affected eye with only one tumor. Yet tumors from both groups were otherwise (histology, cell type, treatment responses, etc.) identical. These observations led Knudson to eventually hypothesize that those children with early-onset retinoblastoma had an inherited mutation in a gene (the retinoblastoma sensitivity gene) and one additional somatic mutation in the other allele inactivated its function leading to a tumor. Children with later-onset retinoblastoma would have to accumulate two mutational events (one spontaneous somatic mutation and a reduction to homozygosity) in the same cell and so developed the tumor rarely, at a later time in life, and only one tumor in one eye would be observed. This hypothesis had the virtue of explaining the observed facts and postulated the existence of tumor suppressor genes.

Within the next decade the retinoblastoma susceptibility gene was cloned (6) and the concept that individuals with early-onset retinoblastoma had inherited forms of the gene while the tumor contained a reduction to homozygosity (7) and both alleles were inactivated was confirmed. Late-onset tumors showed no inherited component, just a somatic mutation in the tumor and a reduction to homozygosity. Introducing the cloned retinoblastoma gene back into cancerous cells and reverting these cells to a normal phenotype proved difficult because overexpression of the retinoblastoma gene in cancerous cells was lethal, but if regulated properly, it appeared to give the normal phenotype (8). When similar experiments were carried out with wild-type clones of the *p53* gene (9), the second gene to be recognized as a tumor suppressor gene, cancerous cells were killed by *p53*-mediated apoptosis while normal cells did not die at low levels of *p53* c-DNAs. In addition, colorectal cancers were shown to harbor mutations in both alleles of the *p53* gene and the *p53* locus showed evidence of a reduction to homozygosity (10). These experiments provided the first clear evidence for the existence of tumor suppressor genes that play a role in human cancers and confirmed the hypotheses that such genes exist.

During this same time, a number of groups were investigating how the DNA tumor viruses caused cancer in animals. Using a genetic approach, it could be shown that viruses, such as SV40 and the human adenoviruses, encoded one or a few proteins that functioned like oncogenes (viral oncogenes) in causing these tumors in animals and transforming cells in culture. These viral oncoproteins were required to maintain the transformed cell and tumorigenic phenotypes. Remarkably, the SV40 oncoprotein, called the large T-antigen, bound to the cellular *p53* and the Rb proteins and inactivated the functions of both proteins in a tumor cell (11). Similarly, the adenovirus E1A oncoprotein bound to the Rb protein, and the E1B-555k oncoprotein bound and inactivated the cellular *p53* protein (11). Finally it was shown that the human papilloma viruses, which are the cause of cervical and penile cancers in humans, encode genes called *E6* and *E7*, which bind to and inactivate the functions of *p53* and Rb in human cancers (12). Thus this group of viruses has selected for the inactivation of two tumor suppressor functions to enhance the replication of these

viruses and in so doing disrupted the cellular process preventing cancers from arising, and these viral-induced tumors result. By the time these observations were recognized, the existence of tumor suppressor genes was established and the list of such genes began to grow (13).

The Tumor Suppressor Genes

Table 3-1 lists a selected number of tumor suppressor genes, their protein's functions, when known, the familial cancer syndrome they may cause, and several of the types of cancers that harbor somatic mutations in these genes (14). The first thing of note is the great diversity of functions of tumor suppressor gene products. There are transcription factors (p53), proteins that negatively regulate transcription factors (Rb), proteins that contribute to the degradation of oncogene functions by activating ubiquitin ligases (APC- β -catenin) or inhibiting ubiquitin ligases that degrade tumor suppressor genes (ARF acts on MDM-2, which degrades p53). There are GTPases (NF-1, TSC-1, 2) that inhibit pro-oncogenic G-proteins and a large number of DNA repair functions (BRCA-1, 2, ATM, MSH2 and MLH1, Franconi anemia genes, etc.). Protein kinases (LKB1) and inhibitors of protein kinases (PTEN, which degrades PIP3, which is a second messenger for several lipid-activated protein kinase, p16-INK4A, which inhibits cdk-2), histone modifications (Men-1 is a histone methylase that silences transcription), and cytoskeletal and adhesion components (E-cadherin, α -catenin, RASSF1, NF-2) are all represented by tumor suppressor genes. What these functions have in common is that they populate signal transduction pathways that participate directly in the cell cycle or confer fidelity to the events in the cell cycle when stress is encountered (such as DNA damage by p53 or hypoxia by p53 and VHL) to avoid errors in the DNA replication and the chromosome segregation process. They prevent cells from entering into cell cycle division under conditions of stress that would result in errors and the development of cancers. In the extreme, they initiate apoptosis to kill clones of cells that may contain or have the potential to obtain these mutations.

The inheritance of a mutated form of these tumor suppressor genes can initiate the formation of a specific set of tumors, commonly at an age much younger than those same tumors arising from spontaneous somatic mutations. Thus the early onset of a cancer in life can be an indication of genetic alterations in a tumor suppressor gene (a mutation or even a single-nucleotide polymorphism [SNP]). An individual harboring a mutation in a tumor suppressor gene may never develop tumors over his or her life time due to incomplete penetrance of the mutation. Only about 50% to 70% of women with *BRCA1* mutations develop breast or ovarian cancers, often depending upon their ethnic group or genetic background (15). SNPs in the genetic background can, in the case of Li-Fraumeni syndrome and *p53* mutations, accelerate the age of onset of a cancer and enhance the number of independent cancers that occur over a life time (16). These SNPs act in the same signal transduction pathway along with mutations in one allele of the *p53* gene. The tissue-specific pattern of somatic mutations in tumor suppressor genes in producing selective cancers remains enigmatic,

and why there are distinct differences in the tumor type with germline mutations compared to somatic mutations also remains to be explained. What is clear is that most of the genetic predispositions that are inherited in the development of cancers are due to tumor suppressor genes and genes involved in DNA repair processes. These predispositions can be in turn modified by SNPs in the genetic background of the host (16).

The p53–Rb Pathway Interconnections

Because tumor suppressor genes act in signal transduction pathways containing multiple oncogenes and tumor suppressor genes, it is best to illustrate the functions of tumor suppressor genes by reviewing some selected examples of these pathways. We can start with the p53 pathway and its interactions with the Rb pathway. The p53 protein responds to a large variety of intrinsic and extrinsic stress signals. These include DNA damage, hypoxia, the shortening of chromosome telomere lengths, spindle poisons, the inhibition of ribosome biogenesis, glucose deprivation, lowering of nucleoside triphosphate pool sizes in cells, and activation of selected oncogenes (myc, ras, E2F-1, β -catenin) or the inactivation of a tumor suppressor gene (APC helps degrade β -catenin, Rb inactivates the functions of E2F-1; 17). One can see from these latter examples that tumor suppressor genes act on (negatively regulate) oncogenes and different signal transduction pathways populated with these genes communicate with each other (the Rb and APC pathways communicate with p53; 18). Each of these stress signals activates the p53 protein. In this case, activation of the p53 protein occurs by an increased half-life (from minutes to hours), an increased concentration of the p53 protein, and the ability of the p53 protein to bind to specific DNA sequences adjacent to a gene that permits the p53 protein to enhance the rate of transcription of that gene. The stress signals are detected and communicated to the p53 protein via a wide variety of enzymes that mediate protein modifications such as phosphorylation, acetylation, methylation, ubiquitination, summolation, and neddylation of the p53 protein and its negative regulator MDM-2 (17,18). MDM-2 is an oncogene (whose gene is amplified in a number of cancers) and a ubiquitin ligase for the p53 protein. A stress signal can result in the modification of the MDM-2 protein, its self poly-ubiquitination resulting in its degradation (19). This in turn results in an increased half-life of the p53 protein. Note that a stress signal in a cell acts on the p53 protein by a posttranslational mechanism so other cellular processes (such as transcription of a damage DNA template) are not essential to activate p53. Once the p53 protein is activated as a transcription factor, it increases the rate of transcription of selected genes that contain a p53 DNA binding site. This begins a program resulting in apoptosis, cellular senescence, or cell cycle arrest (17). Different stress signals result in different modifications of the p53 protein, which in turn result in different transcriptional programs and outcomes for the cell (20). The net result is that a stress signal results in the elimination of a clone of cells that has duplicated itself in an error-prone environment and has decreased fidelity of replication. Just why so many different types of stresses use the p53 pathway is unclear. The fact that the p53 gene and its

Table 3-1 Tumor Suppressor Genes

Gene	Protein Function	Familial Cancer Syndrome	Sporadic Cancers with Mutations
<i>p53</i>	Transcription factor	Li-Fraumeni syndrome	Many (over 50% of all tumors)
<i>RB</i>	Transcriptional regulation	Retinoblastoma, osteogenic sarcoma	Retinoblastoma; osteosarcoma; breast, lung, and bladder carcinoma
<i>WT1</i>	Transcriptional regulation	Wilms tumor	Pediatric kidney cancer
<i>APC</i>	Binds and degrades β -catenin, Wnt signaling	Familial adenomatous polyposis	Colon and stomach carcinoma
<i>NF1</i>	Ras-GAP activity	Neurofibromatosis type I	Astrocytoma, colon carcinoma
<i>NF2</i>	Membrane cytoskeletal attachment	Neurofibromatosis type II	Schwannoma, meningioma, ependymoma
<i>INK4A (p16)</i>	Cdk inhibitor (RB inactivation)	Familial melanoma	Many
<i>ARF</i>	MDM2 antagonist (p53 activation)	Melanoma	Many
<i>VHL</i>	Hypoxia response	von Hippel-Lindau syndrome	Renal cell carcinoma, cerebellar hemangiosarcoma
<i>LKB1</i>	Phosphorylates and activates AMPK to inactivate mTOR	Peutz-Jeghers Syndrome	Lung adenocarcinomas
<i>PTEN</i>	Phosphoinositide-3-phosphatase protein	Cowden syndrome	Glioblastoma, endometrial, thyroid and prostate cancers
<i>TSC1/2</i>	GTPase activation, mTOR inhibition	Hamartoma Tuberous sclerosis	Unknown
<i>BRCA1</i>	DNA damage repair, cell cycle checkpoint control	Familial breast and ovarian cancer	Unknown
<i>BRCA2</i>	Regulation of genes involved in DNA repair and homologous recombination	Familial breast and ovarian cancer	Unknown
<i>FHIT</i>	Nucleoside metabolism	Prostate cancers	Esophageal, stomach, colon and lung carcinoma
<i>DPC4 (Smad4)</i>	Regulation of TGF- β /BMP signal transduction	Familial juvenile polyposis syndrome	Pancreatic carcinoma
<i>PTCH</i>	Transmembrane receptor for sonic hedgehog (shh), involved in early development through repression of action of smoothened	Basal cell nevus syndrome	Basal cell carcinoma
<i>MEN1</i>	Histone methylase	Multiple endocrine neoplasia type 1	Unknown
<i>Beclin 1</i>	Autophagy	Liver (rat and mouse)	Breast and ovarian cancers
<i>ATM</i>	DNA damage sensor (protein kinase)	Ataxia-telangiectasia (T-cell lymphoma)	T-prolymphocytic leukemia and mantle cell lymphoma
<i>MSH2 and MLH1</i>	DNA mismatch repair	Hereditary nonpolyposis colorectal cancer	Endometrial, gastric, ovarian, bladder cancer
<i>E-cadherin (CDH1)</i>	Cell-cell adhesion protein	Familial diffuse-type gastric cancer	Gastric cancer, lobular breast cancer
<i>RASSF1</i>	Cell cycle regulation, apoptosis, and microtubule stability	Unknown	Many
<i>CHK2</i>	Protein kinase (G ₁ checkpoint control)	Li-Fraumeni syndrome	Unknown
<i>FA genes</i>	DNA repair, S-phase checkpoint	Franconi anemia	Acute myelogenous leukemia
<i>NBS1</i>	DNA repair, S-phase checkpoint	Nijmegen breakage syndrome (T-cell lymphoma)	Lymphoreticular malignancies
<i>BIN1</i>	Apoptosis, cell cycle control	Unknown	Breast and prostate cancers

protein form such a central node in a network means that the loss of p53 through mutation makes the cell vulnerable to error-prone division cycles and a higher mutation rate (21). For this reason the p53 gene is found to be mutated in over 50% of all human cancers and individuals with an inherited mutation in one p53 gene always develop cancers in their life times.

One of the genes regulated by the p53 protein is the MDM2 gene. That means that p53 and MDM2 form an autoregulatory feedback loop, where increased levels of p53 protein result in increased levels of MDM2 protein, which in turn lower the levels of p53 (followed by MDM2; 17,18). Thus the levels of p53 and MDM2 in a cell oscillate out of phase after a stress response (22). This could have something to do with the selection of different transcriptional programs (for apoptosis or cell cycle arrest) after stress. For example the activation of p53 in a normal cell in culture most commonly results in a cell cycle arrest. The activation of p53 in a transformed cell in culture commonly results in apoptosis. The mutational activation of some oncogenes, such as myc, ras, E2F-1 or β -catenin), results in the enhanced production of the tumor suppressor protein ARF (23). ARF in turn binds to MDM2 and inhibits its polyubiquitination of the p53 protein (24). This raises p53 levels and results in apoptosis. Similarly the mutational inactivation of the APC tumor suppressor protein, which is required for the degradation of the oncogene β -catenin or the mutation of Rb, which then liberates the E2F-1 oncogene, each result in the enhanced synthesis of ARF by the E2F-1 or β -catenin transcription factors, and this results in p53 activation and apoptosis (Figure 3-1; 23,24). These loops in signal transduction networks interconnect two different signal transduction pathways that initiate cell growth and division with a third p53 stress response pathway.

The Interconnections of the p53 and the IGF-1-mTOR Pathways

One of the major growth and mitogen signaling pathways in a cell is the IGF-1-AKT-1-TSC-mTOR pathways (25). In response to high levels of nutrients, such as glucose and amino acids, insulin is secreted, which results in the production of the insulin-like growth factor -1 (IGF-1). IGF-1 acts to engage its receptor at the cell surface. The cross-linked receptor autophosphorylates itself, which results in the binding of an adaptor and the lipid kinase, PI3 kinase, all localized at the membrane (Figure 3-2). PI3 kinase produces a second messenger, PIP-3 (phosphoinositol 3-phosphate), which in turn activates two lipid-activated (PIP-3) protein kinases, mTOR-ricor and PDK-1. These kinases phosphorylate and activate the AKT-1 kinase. AKT-1 moves from the plasma membrane to the nucleus, where it phosphorylates several transcription factors from the Forkhead family, termed "FOXO transcription factors." The phosphorylated FOXO proteins then leave the nucleus, which results in setting up a transcriptional program that enhances oxidative phosphorylation (efficient energy production), increases the levels of protein folding chaperones (the heat-shock proteins [Hsp's]) and produces proteins that lower the levels of DNA-damaging reactive oxygen species (25).

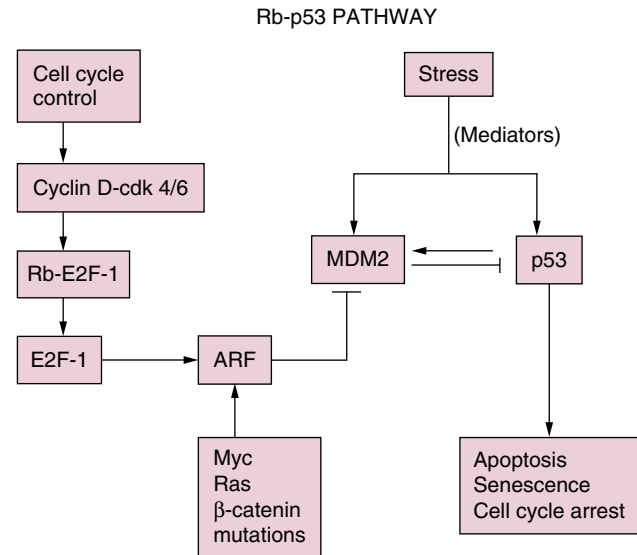
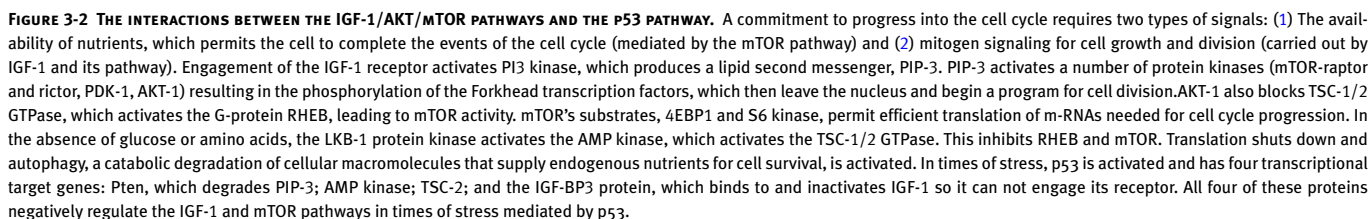


FIGURE 3-1 THE INTERACTIONS BETWEEN THE RETINOBLASTOMA (Rb) AND p53 PATHWAYS. The commitment to progression through the cell cycle begins with increasing concentrations of cyclin D, which activates the CDK-4/6 kinases. This phosphorylates the Rb protein, releasing the E2F transcription factors from Rb-mediated repression. E2F acts to enhance transcription rates of a set of genes required for entry into the cell cycle S phase. E2F also acts to increase the levels of ARF, which raises p53 levels (observed just prior to S phase in the cell cycle) in preparation for stress-induced shut down of cell cycle progression. Thus entry into S phase and a stress signal activates p53 to abort the cell cycle or kill the cell via apoptosis. This is one of the lines of communication between p53 and Rb, two of the most important tumor suppressor genes.

The removal of FOXO from the nucleus also turns off the production of p27, a tumor suppressor protein that inhibits cyclin D-cdk-4/6 protein kinases required for entry into the cell cycle by phosphorylating the Rb protein and liberating E2F-1 protein. Finally the removal of FOXO from the nucleus lowers the signaling for cellular apoptosis, so that the result of this pathway (IGF-1) is a mitogen-driven entry into the cell cycle (25). The IGF-1 pathway must be coordinated with the presence of sufficient nutrients to sustain cell division. This is done by the mTOR pathway (Figure 3-2; 25). The absence of glucose in the environment is signaled to the LKB-1 kinase (a tumor suppressor gene), which signals, via phosphorylation, to the AMP kinase. The low levels of glucose also increase the pool sizes of adenosine monophosphate (AMP) because of the slowing of oxidative phosphorylation and the lowering of energy sources. The presence of high AMP levels and the LKB-1 phosphorylation of the TSC-2 protein, which is in a complex of TSC-1/TSC-2 proteins, increase the GTPase activity of the TSC-1/TSC-2 complex. This in turn inhibits the G-protein Ras homolog enriched in brain (RHEB), which is required for high levels of mTOR-Raptor activity (Figure 3-2). With mTOR-Raptor off, the process of autophagy is activated. Autophagy initiates the formation of double-membrane vesicles in the cell cytoplasm, which engulf ribosomes, proteins, carbohydrates, and lipids in the cell and deliver them to the endosome for degradation. This liberates nutrients by catabolic processes and maintains a cell for a period of time during starvation of exogenous nutrients. With time, the cell volume get smaller and



of selected mRNAs for ribosomal biogenesis. Thus, mTOR reciprocally regulates translational controls and autophagy in response to the levels of glucose and amino acids in the medium (25,28).

The IGF-1 and mTOR pathways are coregulated via the tumor suppressor genes TSC-1/2, and this coordinates two critical signals for growth (mTOR) and mitogen-driven cell cycle division. Both signals must be positive for a cell to commit to cell division. But what happens when both signals are positive for growth and division and a stress signal appears that will decrease the fidelity of the division process? Here p53 is activated and the transcription rates of four p53-regulated genes increase (25,28,29). All four of these genes play a role in the IGF-1 and mTOR pathways, and all four of these gene products negatively regulate the IGF-1 and mTOR pathways. p53 induces the synthesis of PTEN, a PIP-3 phosphatase that degrades PIP-3 to PIP-2, which no longer activates mTOR rictor, PDK-1 AKT-1, or mTOR Raptor. In addition, the loss of AKT-1 activity increases the TSC-1/TSC-2 activity and lowers mTOR activity. p53 also regulates the increase in TSC-2 concentration, which has the same impact. p53 also increases the concentration of the β subunit of AMP kinase (29). AMP kinase is a trimeric protein where the α subunit is the kinase catalytic subunit, the γ subunit is the AMP binding and regulatory subunit, and the β subunit coordinates the two other proteins. Increasing the β subunit increases the AMP kinase activity, increases the

higher levels of TSC-1/2 complex and activity, and shuts down mTOR. The fourth p53-regulated protein to increase after a p53-mediated stress signal is the IGF-BP-3 protein, which binds to free IGF-1 and prevents it from interacting with the receptor, shutting down AKT- signaling (Figure 3-2; 25). Thus p53 shuts down this critical growth response pathway in the event of a stress that would lower the fidelity of cell division. All four of these p53-regulated genes that modulate down the IGF-1 and mTOR pathways do so in a tissue-restricted fashion (25,29). The tissues where most or all of these genes act are the tissues that require insulin for glucose uptake (fat, muscles, liver, intestine, kidney; 29). These types of restricted regulatory patterns of expression can help to explain some of the tissue preferences of oncogenes or tumor suppressor genes in cancers. Note in Figure 3-2 that there is an interesting positive-feedback loop between p53 and the IGF-1 pathway. A p53 stress activation produces PTEN, which inhibits the AKT-1 kinase. The AKT-1 kinase can phosphorylate and activate the MDM-2 protein, which lowers p53 levels and activity: Low AKT-1 activity decreases the MDM-2 activity, which increases p53 functions. This p53–PTEN–AKT-1–MDM-2 loop positively regulates p53 activity after stress and higher levels of p53 favor an apoptotic response (18).

Conclusion

These four pathways (Figures 3-1 and 3-2) contain many oncogenes (MDM-2, PI3 kinase, AKT-1, cyclin D, E2F-1) and tumor suppressor genes (*p53*, *PTEN*, *TSC1*, *TSC2*, *LKB1*, *Rb*, *ARF*) that regulate each other and form the interconnections between pathways (25). Together they coordinate the division process and its requirements for mitogens and nutrient growth signals with stress signals that influence the fidelity of cell division. Failure of any of these homeostatic processes can lead to cancerous growth and the loss of apoptotic control over these mistakes. The system has many redundancies, which should provide fail-safe controls, but the number of cells in the body and the number of cell divisions over a life time are a real challenge to any design. We can readily understand why only one gene in a specific pathway might contain a mutation because two mutations in the same pathway would add little to the disruption of that pathway. Less clear is the tissue specificity of the pattern of oncogene mutations and tumor suppressor gene mutations observed with the inherited route or somatic mutation. The field is just at the beginning of the process of collecting these data, understanding this pattern of gene mutations for each tumor type, and ascribing a functional consequence to that pattern. This will be an important area for future research. Just how SNPs modify the penetrance of tumor suppressor mutations or modulate the efficiency of a signal transduction pathway is also a new area of research that has a productive future (16,30,31). It has become much easier to understand human genetic processes now that we have the complete sequence of the human genome and have begun to assemble functional signal transduction pathways. Such pathways provide us with a better understanding of epistatic relationships between genes and how one gene and its SNPs can modify or suppress another gene. The genetics of cancers in humans will become more facile over the next years.

Equally interesting will be to track down the environmental variables that contribute to cancers and this will lead to better prevention strategies. The types of somatic mutations in tumors and the tissues affected by mutagens has led to the new field of tumor archeology where the nature of the mutation or base change leads one to the mutagen and ultimately the source of the carcinogen. Here the types of mutations and the DNA sequence contexts in the *p53* gene obtained from lung cancers and liver cancers have identified benzo[a]pyrene in cigarette smoke (32) and aflatoxin B1 in fungal contamination of peanuts (33) as the mutagens that caused these *p53* mutations. Indeed a database of *p53* mutations from many tissues (34) shows this kind of tissue preferences for mutational hot spots as well as tissue-independent hot spots. Epidemiologic studies with environmental exposures to mutagens and a good knowledge of the gene products that deal with these stresses in the environment (like p53 and DNA damage) are now beginning to appear. For example Zhang and his colleagues (35) have examined the impact of the genotype of SNPs in the *MDM2* and *p53* genes (these genes are epistatic to each other) along with smoking on the odds of developing lung cancer in China. The SNP 309 G/G genotype has been shown to result in high *MDM2* levels and low p53 levels in people. The p53 codon72 pro/pro SNP has been shown to have lower p53 activity for inducing apoptosis than the alternative allele. The G/G SNP in the *MDM2* gene (SNP 309) had an odds ratio of developing lung cancer of 1.83. The codon 72, pro/pro SNP had an odds ratio of 1.47. The combined odds ratio *MDM2* G/G and *p53* pro/pro in the population of lung cancer patients was 4.56, which shows the synergistic and epistatic impact of these two proteins that act on each other. The odds ratio of those patients with a genotype of *MDM2* G/G and *p53* pro/pro who also were smokers was 10.41, demonstrating the impact of an environmental stress system on the less-efficient *MDM2* and *p53* alleles in the population when compared with individuals with the more active *p53* alleles (35). Clearly these epidemiologic observations are most meaningful when we understand the molecular biology of these signal transduction pathways.

The concepts of tumor suppressor genes have led to our understanding of the inherited basis of cancers in humans. These mutations have also led to a new understanding of biologic processes that are essential for cell division, programmed cell death, and an enhanced fidelity of the cellular duplication process. All organisms have learned to deal with environmental stresses, changing environments, and nutrient deprivation. They have evolved elaborate mechanisms to wait for better times before having offspring or eliminating those offspring that are not good copies of themselves. The p53 gene in flies and worms acts in the germ line to eliminate clones of eggs or sperm with DNA damage (36,37). This function continues in mice (and probably humans; 38) but vertebrates have also adapted the p53 pathway for the surveillance of somatic cells that duplicate many times over a life time. Worms and flies are born with no more programmed somatic cell divisions. The vertebrate strategy is one of constant somatic tissue regeneration and therefore the need for the p53 pathway. While the p53 gene and its protein are not essential for life (a mouse with no p53 genes is alive and develops many cancers; 39), it is essential for the high fidelity required by the duplication process of all living organisms.

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DNA Repair Pathways and Human Cancer

DNA repair is central to the field of cancer biology and has important implications for cancer diagnosis and treatment. Cancer cells are often deficient in a normal DNA repair function, and this deficiency allows the tumor to develop genomic instability (1,2). With defective DNA repair, the tumor cell can break and re-form chromosomes, generate new oncogenic fusion genes, disrupt tumor suppressor genes, amplify drug-resistance genes, and progress to a more malignant state. A DNA repair deficiency also accounts for the enhanced sensitivity of tumor cells to genotoxic agents, such as ionizing radiation and genotoxic chemotherapy. A thorough knowledge of DNA repair mechanisms in normal and cancer cells may therefore lead to better clinical management of cancer.

The Spectrum of DNA Damage

Spontaneous DNA Damage

To understand the process of DNA repair, one must first consider the wide range of DNA-damaging events in a cell. DNA may undergo spontaneous damage, such as deamination of cytosine or spontaneous hydrolysis of the phosphodiester backbone. DNA may develop mismatched bases, perhaps resulting from the deployment of an error-prone DNA polymerase during S-phase progression. DNA may be attacked by reactive oxygen species (ROS). Indeed, some of the most sophisticated DNA repair mechanisms in a cell are mechanisms that cope with the removal of oxidative DNA lesions.

Of particular relevance to cancer is the DNA damage from alkylating agents or ultraviolet (UV) light or ionizing radiation (IR). DNA damage resulting from these environmental agents can lead to heightened mutagenesis and oncogenesis. Also, many of these agents themselves have anticancer activity. Thus, DNA-damaging agents can cause human cancer but, ironically, are among the primary means available to clinicians for treating cancer. Accordingly, some chemotherapeutic agents have effective anticancer activity in the short run but are responsible for causing secondary cancers in the long run.

DNA Damage from Antineoplastic Therapeutic Agents

Most anticancer agents function by directly damaging DNA. Effective anticancer drugs include monofunctional alkylating agents (cyclophosphamide, 1,3-bis[2-chloroethyl]-1-nitrosourea [BCNU]), bifunctional alkylating agents, (cisplatin, carboplatin, oxaliplatin), and DNA-intercalating agents (adriamycin). In addition, IR and the radiomimetic agent, bleomycin, can cause double-strand breaks in DNA directly. Bleomycin is a small glycopeptide that chelates ferrous ion and binds to specific sequences of double-stranded DNA containing pyrimidine repeats. In the presence of oxygen, bleomycin generates a local high concentration of hydroxyl radicals capable of causing local double-strand breaks. Other drugs, such as the topoisomerase inhibitors, etoposide, and camptothecin, can lead to the accumulation of DNA damage. Thus, known anticancer agents can generate a wide range of DNA damage, including damaged bases, single-strand breaks, and double-strand breaks.

It is important for oncologists to bear in mind that anticancer drugs generate their cytotoxic effects through DNA damage. First, effective anticancer protocols often include a combination of chemotherapeutic agents and IR. Together, these agents cause a broader spectrum of DNA damage in the tumor than single-agent therapy (monotherapy). This broad spectrum may contribute to the synergy observed with these agents. Second, chemotherapy combinations are often chosen to limit toxicity to normal tissue. Agents that generate the same class of DNA damage (such as IR and bleomycin, which both generate double-strand breaks) may have enhanced toxicity compared with other combinations. Some newer classes of drugs inhibit normal DNA repair processes. These so-called chemosensitizers may be particularly effective when used in combination with a more traditional cytotoxic, DNA-damaging drug. A combination of a DNA repair inhibitor and a direct DNA-damaging agent can also result in significant toxicity to normal tissue. One way to limit this toxicity would be to deliver one agent such as the chemosensitizer systemically, but to deliver the other agent, such as IR, locally to the tumor.

DNA Repair

DNA repair is strictly defined as the cellular responses that are associated with the restoration of the normal base-pair sequence and structure of damaged DNA. As described in the following sections, there are six primary DNA repair pathways, and each pathway is composed of a series of biochemical events leading to the sensing, excision, and restoration of normal DNA sequence.

The Systematic Study of DNA Repair

It is instructive to consider the history of DNA repair research as it relates to cancer biology. Early studies of DNA repair evolved from the study of normal DNA replication and metabolism. These early studies relied heavily on the use of damaged DNA templates as substrates for the purification of DNA repair enzymes. Such templates were incubated with cell-free extracts, and the recovered DNA was analyzed for specific incision and excision events. Not surprisingly, these assays uncovered many of the pertinent endonuclease and exonuclease activities required for DNA repair. It has become increasingly apparent that DNA repair proteins are assembled in protein–protein complexes, such as the excision repair complex or the mismatch repair complex. Still, the regulatory networks and relevant posttranslational modifications of DNA repair proteins (i.e., phosphorylations and ubiquitinations) were largely missed by these early biochemical studies.

The study of inherited human DNA repair disorders also contributed greatly to the recognition of the six major DNA repair pathways (see following section). These studies depended on the establishment of mutant human cell lines derived from patients with genetic diseases. For instance, in 1968, James Cleaver isolated fibroblast lines from humans with the disease, xeroderma pigmentosum (XP; 3,4). Importantly, these lines retained their UV light-hypersensitivity phenotype and have been invaluable tools for somatic cell fusion, complementation analysis, and expression cloning of XP genes. Subsequently, other investigators were able to establish mutant cell lines from humans with other DNA repair disorders such as ataxia-telangiectasis (A-T), Fanconi anemia (FA), and Nijmegen breakage syndrome (NBS; 5). These cell lines continue to be used extensively as models of human cancers that also lack the relevant DNA repair pathways.

The study of model organisms has contributed greatly to our understanding of DNA repair processes. For instance, investigators isolated mutants in the yeast, *Saccharomyces cerevisiae*, which were hypersensitive to UV light, IR, or DNA cross-linking agents. In many cases, the genes that were mutated in these yeast strains cooperated in common DNA repair and DNA damage response pathways. IR-induced double-strand breaks in DNA are normally repaired by the DNA repair process of homologous recombination. Accordingly, many of the relevant genes corresponding to these mutant strains and required for normal homologous recombination (HR) repair were first isolated in yeast. Thereafter, the human homologues of these genes were identified. Other model organisms and cell lines have been especially important in the identification of

genes involved in DNA repair pathways, such as mismatch repair (6) and translesion DNA synthesis (7). Among the most useful model systems for studying DNA repair are the *Caenorhabditis elegans* (8) and chicken (DT40) genetic systems (9).

In the postgenomic era, and following the identification of a large number (perhaps 130) of distinct DNA repair proteins (10), investigators have turned to x-ray crystallography for a detailed understanding of DNA repair protein interaction with damaged DNA. The structures of many endonucleases, helicases, and ligases are now available, providing the opportunity for computer-assisted drug development (CADD) of DNA repair enzyme inhibitors. Also, mass spectrometry has been used to identify critical post-translational modifications of DNA repair proteins. These modifications appear to be critical to the proper localization and assembly of DNA repair complexes around sites of DNA damage. The modifications may also regulate the intrinsic catalytic activity of the repair complexes. These protein modifications also can be used as surrogate markers, or biomarkers, of DNA repair activity in a given tumor type (see following sections).

The Six Major DNA Repair Pathways in Human Cells

As described previously, the combination of (1) biochemistry with damaged DNA templates, (2) human mutant cell lines with genetic deficiencies of DNA repair, (3) genetics of yeast mutants with IR or UV sensitivity, and (4) structural studies of DNA repair proteins has led to the establishment of six major DNA repair pathways. These pathways are base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), nonhomologous end joining (NHEJ), and translesion DNA synthesis (TLS).

There is also considerable redundancy in the function of the DNA repair pathways. When one pathway is disrupted, another pathway can partially compensate, especially if the second pathway is up-regulated. For instance, a cell that is deficient in HR repair may depend more on the error-prone repair NHEJ pathway for the repair of double-strand breaks. Also, thymine dimers, which are generated by UV light exposure, can be repaired by NER repair or bypassed and effectively ignored by TLS polymerases. In some cases, the absence of one DNA repair pathway results in a hyperdependence on one or more other DNA repair pathways (11,12). This so-called synthetic lethality among DNA repair pathways has important implications for the design of new anticancer drugs (see following paragraphs).

The six DNA repair pathways are not constitutively activated, but instead they are highly regulated. The pathways are often activated at discrete times in the cell cycle. For instance, HR repair and TLS repair are active during the S phase of the cell cycle. Also, the DNA repair pathways are differentially active in various tissues and cell types. For instance, HR and TLS are more active in rapidly growing cells, such as hematopoietic cells, whereas NHEJ is more active in postreplicative cells. Accordingly, absence of a particular DNA repair pathway may be particularly disruptive to the growth and survival of some normal tissues and some cancers. Here is a brief description of the six DNA repair pathways, with an emphasis on the enzymes in the pathways and the preference for DNA lesions repaired.

Base Excision Repair

Base excision repair (BER) has been reviewed by Wilson (13). BER is used by the cell to correct damaged DNA bases or single-strand DNA breaks. These lesions often result from spontaneous DNA damage (DNA deamination or hydroxylation of bases) or by exposure to environmental alkylating agents. In this pathway, damaged bases are removed by one of at least ten DNA glycosylases, the resulting apurinic/apyrimidinic (AP) sites are processed first by the Ape1 AP endonuclease, leaving a 5' deoxyribose-phosphate; then by an AP lyase activity leaving a 3'-elimination product. Single-strand breaks are then filled in by a DNA polymerase, either with a single nucleotide or with a longer repair patch, followed by ligation. A schematic representation of BER is shown in Figure 4-1.

Mismatch Repair

MMR has been reviewed (6). MMR rapidly removes mispaired nucleotides that result from replication errors and is involved in the detection and repair of DNA adducts such as those resulting from platinum-based chemotherapeutic agents. Initially, the heterodimeric MSH complex recognizes the nucleotide mismatch, followed by its interaction with MLH1/PMS2 and MLH1/MLH3 complexes. Several proteins participate in the process of nucleotide excision and resynthesis. Tumor cells deficient in mismatch repair have much higher mutation frequencies than normal cells and exhibit microsatellite instability, a genomic biomarker of the underlying defect. At least six genes, *MSH2*, *MLH1*, *PMS2*, *MSH3*, *MSH6*, and *MLH3*, are involved in mismatch repair. A schematic representation of MMR is shown in Figure 4-2.

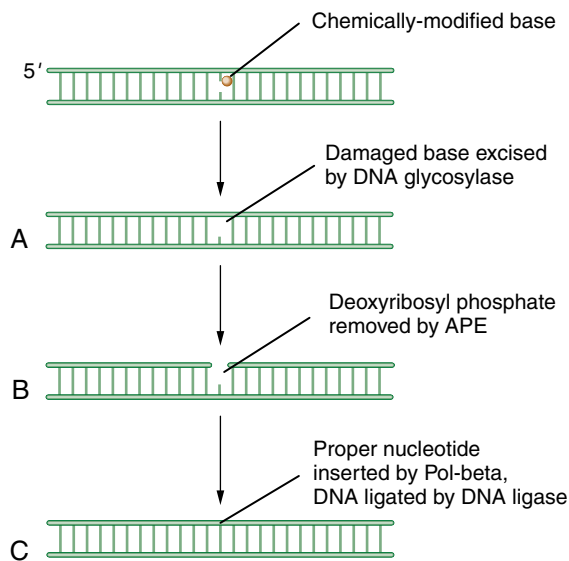


FIGURE 4-1 SCHEMATIC DESCRIPTION OF BASE EXCISION REPAIR (BER). BER is focused on small DNA lesions, often from endogenous sources, resulting in minor helix distortions. Initially, the lesion is recognized by one of the cellular DNA glycosylases, which cleaves the covalent bond between the abnormal base and the deoxyribose sugar (A). This cleavage leaves a so-called apurinic/apyrimidinic (AP) site. Next, the apurinic endonuclease (APE) is recruited to cleave the phosphodiester backbone of the DNA (B). Finally, an error-free polymerase, Pol- β , is engaged to replace the normal nucleotide, followed by DNA ligation (C) and restoration of the normal double-stranded DNA sequence.

Nucleotide Excision Repair

Nucleotide excision repair (NER) acts on a variety of helix-distorting DNA lesions, caused mostly by exogenous sources that interfere with normal base pairing. This pathway may be particularly important in the response to adduct-forming chemotherapeutic agents such as platinum-based chemotherapy (14). The primary function of NER appears to be the removal of damage, for example pyrimidine dimers, which are induced by UV light. Members of the NER pathway include the XPA, XPB, XPC, XPD, XPE, and XPG proteins. Two other NER proteins, XPF and ERCC1, are especially important for the processing of DNA cross-link repair. Studies indicate that monitoring the levels of these proteins in tumors may provide important biomarkers for predicting cross-linker drug sensitivity.

As for the other DNA repair pathways, these proteins cooperate to recognize and excise the damaged nucleotides and resynthesize and ligate the damaged DNA strand. In the process of NER, initially a DNA-binding component, the DDB, binds to sites of damaged DNA, such as cyclopuridine dimers

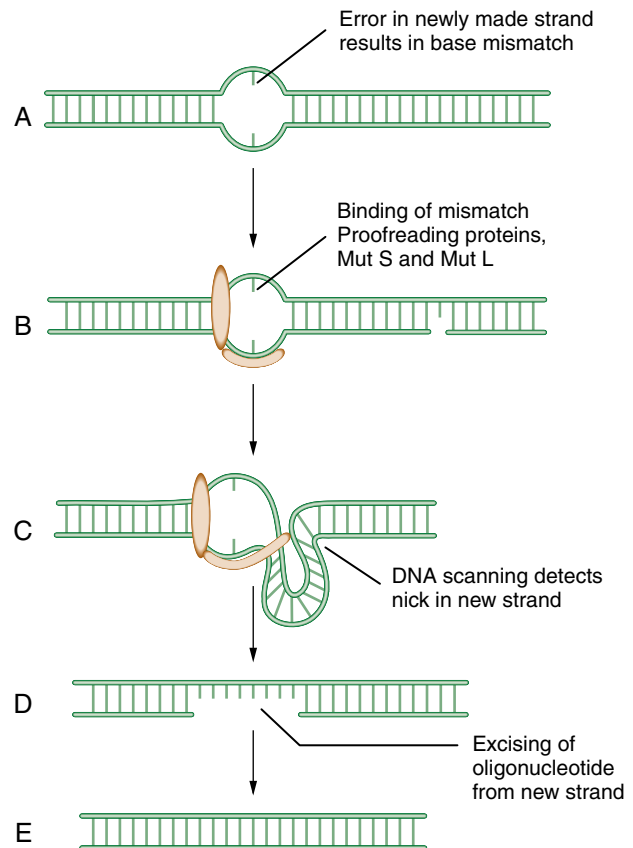


FIGURE 4-2 SCHEMATIC MODEL OF MISMATCH REPAIR (MMR). Mismatch repair proteins function by sensing, binding, and repairing mistakes made during DNA replication. These mistakes include misincorporated bases and errors made during replication of microsatellite sequences. MutS can bind to the mismatch and generate a kink in the DNA. This allows MutL to scan the DNA for a nearby single-strand nick in the newly replicated DNA. MutL then identifies, cleaves, and removes an oligonucleotide patch from the newly replicated strand (D). This allows replication and the insertion of the proper DNA base at the site of the former mismatch. Mutations in the human genes encoding homologues of these bacterial proteins play a critical role in the inherited disease, hereditary nonpolyposis colorectal cancer (HNPCC).

or 6–4 photoproducts. The DDB consists of DDB1 and DDB2. Mutations in the *DDB2* gene cause the genetic complementation group, XPE. DDB is part of a ubiquitin E3 ligase that polyubiquitinates XPC. Polyubiquitination of XPC results in enhanced DNA binding. This binding sets the stage for the downstream binding of the entire excision repair complex, TFIIH, thus leading to excision of the damaged bases.

Eukaryotic NER includes two major branches, transcription-coupled repair (TCR) and global genome repair (GGR). GGR is a slow, random process of inspecting the entire genome for injuries, whereas TCR is highly specific and efficient and concentrates on damage-blocking RNA polymerase II. The two mechanisms differ in substrate specificity and recognition, and hence the enzymes involved are important nodal points for post-translational modifications. A schematic representation of NER is shown in Figure 4-3.

Homologous Recombination Repair

DNA double-strand breaks (DSBs) can be caused by many different environmental factors, including reactive oxygen species, IR, and certain antineoplastic drugs, such as bleomycin, anthracyclines, and topoisomerase inhibitors. Alternatively, DSBs can result from endogenous factors, especially during normal S-phase progression. Failure to repair DSBs can lead to a number of consequences, including mutations, gross chromosomal rearrangements and other aberrations, and eventually cell death. HR is a process by which DSBs are repaired through the alignment of homologous sequences of DNA and occurs primarily during the late S to M phase of the cell cycle. Initially the RAD50-MRE11-NBS1 complex, which possesses a 3'–5' exonuclease activity, exposes the 3' ends on either side of the DSB, a process that may also require *BRCA1*. The 3' advancing strand from the damaged chromosome

then invades the complementary sequence of the homologous chromosome, and the breast cancer susceptibility protein, *BRCA2*, and the single-strand DNA binding protein, *RAD51*, are required for the process. The 3' end of this strand is then extended by an HR polymerase by reading off this complementary sequence. After replication has extended past the region of the DSB, the 3' end of the advancing strand returns to the original chromosome and replication continues. A schematic representation of HR is shown in Figure 4-4. HR repair is especially important in the repair of DSBs and DNA interstrand cross-links. Since some tumors, particularly breast and ovarian tumors, are defective in HR repair, drugs that cause these lesions may be particularly effective in this setting.

Nonhomologous End Joining

Nonhomologous end joining (NHEJ) has been reviewed by Lieber et al. (15). NHEJ is another major pathway of repairing DSBs. Similar to HR, this pathway is important in the repair of agents that result in DSBs such as IR, bleomycin, topoisomerase II poisons, and anthracyclines. The DNA-dependent protein kinase (DNA-PK) consists of the catalytic subunit (DNA-PKcs) and the regulatory subunit (the Ku70/Ku80 heterodimer). The DNA-PKcs subunit is a serine/threonine kinase that belongs to the phosphatidylinositol-3 kinase family. The Ku80/Ku70 heterodimer (Ku) exhibits sequence-independent affinity for double-stranded termini and on binding to DNA, ends recruits and activates the DNA-PKcs catalytic subunit. Additional proteins are required for the completion of NHEJ, including the artemis protein and DNA ligase IV. Importantly, NHEJ is an error-prone repair pathway. Since the process does not use a complementary template, the fusion of the blunt-ended DNA duplexes may result in deletion or insertion of base pairs. A schematic representation of NHEJ is shown in Figure 4-5. NHEJ has a normal function in immune cells to generate diversity at the immunoglobulin and T-cell receptor gene loci.

Translesion DNA Synthesis

The process of TLS is another mechanism for dealing with thymine dimers and bases with bulky chemical adducts. At a DNA replication fork, DNA adducts may cause a replicative polymerase, such as DNA polymerase Δ , to stall. Cells have, therefore, developed sophisticated mechanisms for switching off the replicative polymerase and switching on alternative polymerases (i.e., a polymerase such as Pol η , which will replicate past certain DNA lesions with high fidelity; 16). Interestingly, human cells have at least 15 DNA polymerases, although the situations and mechanisms of their deployment are largely unknown (17). Cancer may have a heightened dependence on one of the error-prone TLS polymerases, such as polymerases β or κ , accounting for high rates of mutagenesis. A schematic representation of TLS is shown in Figure 4-6.

Examples of Redundancy in DNA Repair Pathways

Specific DNA repair pathways can antagonize the activity of anti-cancer agents. The status of a particular DNA repair pathways

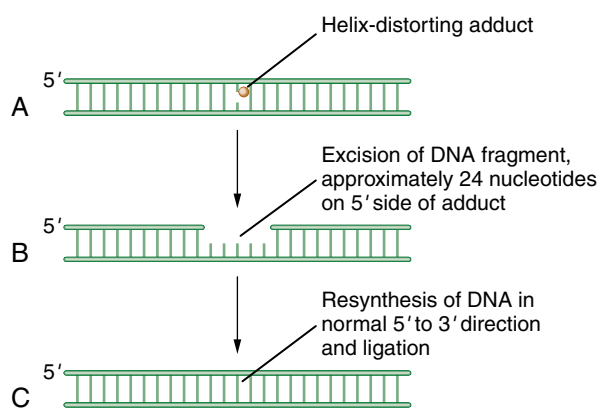


FIGURE 4-3 SCHEMATIC MODEL OF NUCLEOTIDE EXCISION REPAIR (NER). A: NER is invoked when a base is modified by a larger helix-distorting lesion, such as an UV-generated thymine dimer. Initially, the bulky lesion is recognized by a sensor complex, including the XPE protein (also known as DDB2). This protein is part of an ubiquitin-conjugating complex, containing Cul4A and DDB1. The complex polyubiquitinates XPC, allowing for the recruitment of the excision repair complex. Next a patch of nucleotides is excised from the damaged DNA. In general, the excision occurs approximately 24 nucleotides 5' to the damaged base and three nucleotides to the 3' side (B). Finally, new DNA polymerization can occur, and the repaired DNA is ligated (C). The NER complex is a large multisubunit complex. Mutations in genes encoding subunits of this complex underlie the human disease, xeroderma pigmentosum (XP). The complex also contains proteins that can recognize and remove bases with large bulky adducts such as those generated by polycyclic hydrocarbons and aflatoxin B1.

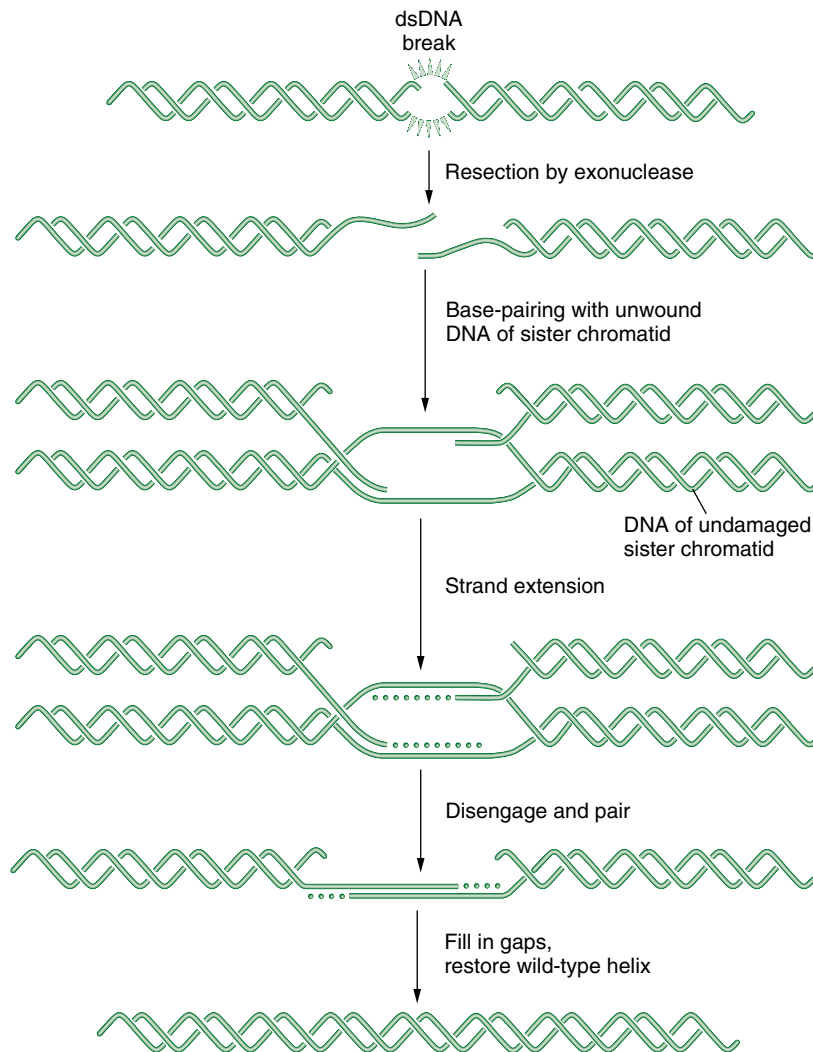


FIGURE 4-4 SCHEMATIC REPRESENTATION OF HOMOLOGOUS RECOMBINATION (HR). HR repair is required for the normal repair of double-strand breaks as well as covalent interstrand DNA cross-links. Initially, the double-strand break (DSB) is recognized by a sensor (A). One of the early events is the binding of phosphorylated histone 2AX to chromatin areas flanking the DSB. Next, an unknown exonuclease functions to trim back the DNA, leading to 3' single-strand overhang of DNA. These single-strand sequences are rapidly coated with Replication Protein A (RPA), followed by replacement with the HR protein, RAD51 (B). Next, the RAD51-coated single-strand DNA "invades" the normal sequence of the sister chromatid (or chromosome homologue) (C). This strand invasion allows the 3' end of the broken helix to synthesize DNA past the site of the DSB. Once this process occurs, the two sister chromatids can disengage, with the use of an enzyme complex referred to as a "resolvase" (D). Ultimately, a normal DNA sequence is regenerated. Interestingly, some tumors have defects in HR repair, such as *BRCA1*- or *BRCA2*-deficient breast cancers. These tumor cells have prolonged time periods with unrepaired DSBs, thus leading to chromosome-translocation events and a more malignant phenotype. Alternatively, the defective HR repair results in hyperdependence on the more error-prone nonhomologous end joining (NHEJ) mechanism. (Adapted from Weinberg RA. *The Biology of Cancer*. Garland Science, Taylor and Francis Group, 2006).

in a tumor may therefore predict the best antitumor therapy. As described previously, at least two DNA repair pathways are dedicated to the removal of DNA bases modified by monofunctional alkylating agents: the BER and NER pathways. BER can cleave the bond linking the modified base to the deoxyribose. NER, in contrast, will remove the entire modified nucleotide, along with a small stretch of surrounding nucleotides. In either case, the undamaged DNA can be used to synthesize the normal DNA sequence, followed by ligation of the segments.

Cancer cells have other mechanisms for coping with modified bases. One enzyme, MGMT (O⁶-methylguanine-DNA methyltransferase), is capable of catalyzing the reversal of the chemical modification. Interestingly, this enzyme is switched off by MGMT gene promoter methylation in some solid tumors (gliomas and colorectal tumors) accounting, at least in part, for the hypersensitivity of these tumors to some monofunctional alkylating agents.

In addition, damaged bases in the DNA can be bypassed through the use of TLS. Through this mechanism, the modified lesions are sensed, the normal replicative polymerase is removed from the replication fork, and a new polymerase is invoked to bypass the lesions. Rapid TLS (damage avoidance) is essential for a cell to transverse S phase rapidly, without succumbing to replication arrest

and apoptosis. Interestingly, TLS is an error-prone process, however, and the promiscuous use of TLS by cancer cells may result in their increased mutation frequency (see following sections).

Some of the 15 variant polymerases can extend a nascent DNA strand past a thymine dimer or past a bulky DNA lesion. Other variant polymerases can replace a single nucleotide at the site of an unpaired base. One of these variant polymerases, referred to as Pol η , is mutated in the autosomal recessive human disease, XP-variant (xeroderma pigmentosum variant). Absence of the Pol η enzyme results in UV light hypersensitivity, an inability to replicate past thymine dimers, and a predisposition to squamous cell cancers (Table 4-1).

The variant polymerases exhibit a variable level of fidelity. Important unanswered questions in the TLS research field include (1) what are the circumstances and mechanisms for recruiting the variant polymerase to a specific damaged DNA site; and (2) are any of these variant polymerases overexpressed or dysregulated in cancer, accounting for the elevated mutation frequency of solid tumors?

IR causes DSBs and a wide range of oxidative DNA damage. Two redundant DNA repair pathways, HR repair and NHEJ repair, are particularly adept at dealing with DSB damage in a cancer cell. In clinical oncology, some tumors that have defects in

FIGURE 4-5 SCHEMATIC REPRESENTATION OF NONHOMOLOGOUS END JOINING (NHEJ). NHEJ is an error-prone alternative to homologous recombination (HR) repair that can also be used to repair double-strand breaks. Since NHEJ does not use a homologous DNA template, such as a sister chromatid or a homologous chromosome, it often results in the insertion or deletion of new nucleotides at the fused DSB junction. In NHEJ, the DSBs are coated by the blunt-end-binding protein, Ku. In some cases, the blunt ends may be brought together by limited microsequence homology. The enzymes DNA-PK, XRCC4, Artemis, and DNA ligase IV are required for the successful religation of the free ends. Interestingly, NHEJ appears to be the repair mechanism used for the cleavage and religation of immunoglobulin (Ig) gene variable regions; hence, the error-prone religation adds to the diversity of the somatically generated Ig gene repertoire. Germ-line mutations in some NHEJ genes, such as DNA-PK and Artemis, results in an inherited defect in NHEJ and a severe combined immunodeficiency syndrome.

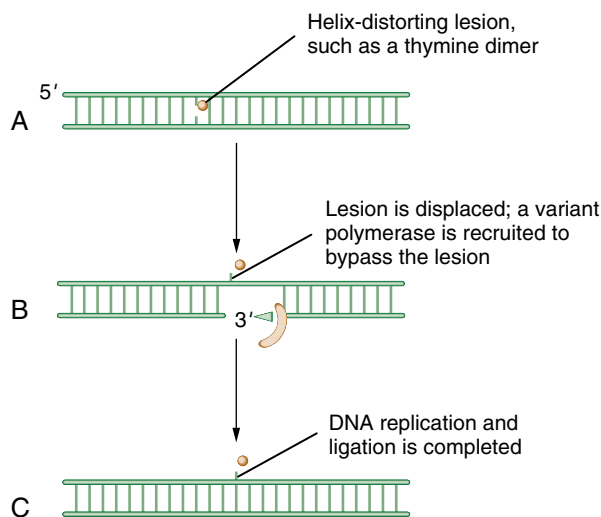
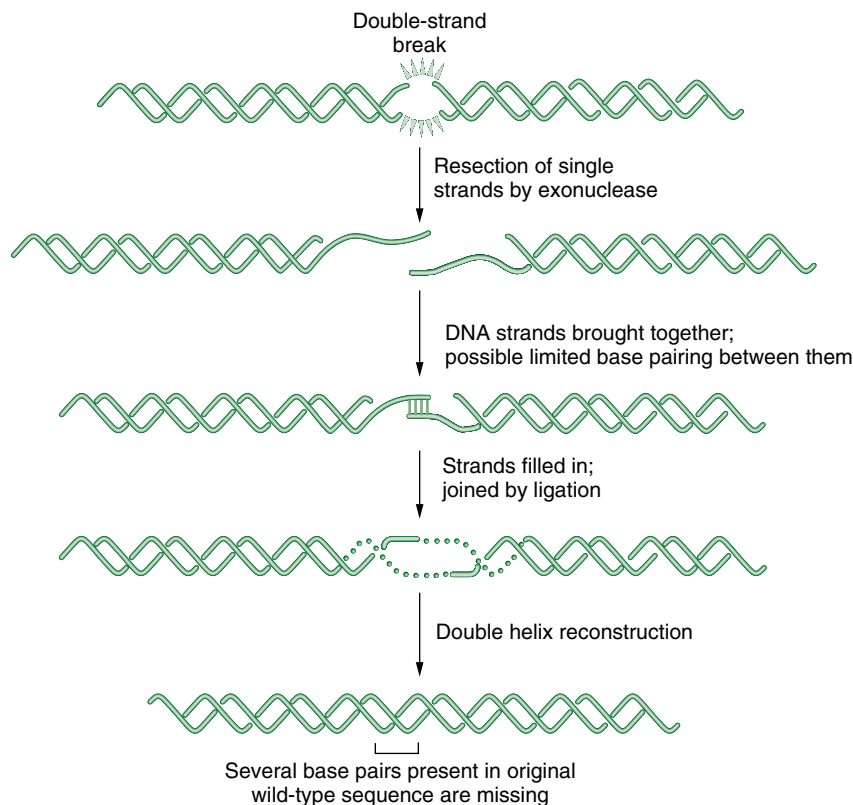


FIGURE 4-6 SCHEMATIC MODEL OF TRANSLESION SYNTHESIS REPAIR (TLS). A: TLS is not a DNA repair pathway per se, it is a mechanism of DNA damage bypass. In this process, an advancing replication fork encounters a damaged DNA base. While the replicative polymerase (the Pol- Δ complex) cannot read through the damaged base, a variant polymerase such as Pol eta, can bypass the lesion. Cells have developed sophisticated mechanisms for switching polymerases (B). For instance, in response to UV damage and the generation of a CPD (cyclopurine dimer), the processivity factor, PCNA, becomes monoubiquitinated by RAD18. Modified PCNA now excludes Pol- Δ binding and has preferred binding for Pol eta. Pol eta is recruited, and it has the ability to “read through” the damaged base and insert the proper nucleotide (i.e., AA residues are replaced opposite the TT residues of a thymine dimer). Less is known about the regulation of TLS than of other DNA repair pathways. Depending on the kind of DNA damage, it is becoming increasingly clear that there are biochemical “switching” mechanisms for recruiting one of the other 12 TLS polymerases, as needed.

these DNA repair processes are particularly sensitive to the cytolytic activity of IR. Also, radiation resistance can emerge through the induction of these DNA repair activities in treated tumor cells. Tumor cells that grow in a more hypoxic environment may also be more resistant to the killing effect of IR, perhaps due to the decrease in oxidative damage generated in these cells.

Regulation of the Six DNA Repair Pathways

As described previously, the major proteins involved with DNA repair include sensory (DNA binding) proteins, enzymes that remove damaged bases, and enzymes that restore the normal DNA sequence. A large number of regulatory enzymes also control each DNA repair pathway. These enzymes are required for switching on and switching off DNA repair, as needed by the cells. Regulatory enzymes, such as helicases, serve to load DNA repair complexes at the sites of DNA damage. Other regulatory enzymes, such as topoisomerases, serve to unwind damaged DNA to facilitate DNA repair complex assembly, loading into chromatin, and disassembly.

A major subclass of regulatory enzymes adds critical post-translational modifications to DNA repair enzymes. For instance, in BER, a sumoylating enzyme modifies one of the glycosylases, TDG, thereby enhancing the activity of the glycosylase in removing damaged bases (18,19). In NER, an E3 ligase complex (Cul4A, DDB1, DDB2) activates the polyubiquitination of the XPC protein. This XPC modification is a necessary event for the downstream activity of the NER complex (20). In TLS, an E3 ligase, RAD18, monoubiquitinates the DNA processivity factor, PCNA,

Table 4-1 The Six Major DNA Repair Pathways

DNA Damage Repair Pathway	Function	Examples of Gene Mutation	Examples of Altered Expression of a Normal Gene	Effect of Loss of Pathway on Clinical Response
Base excision repair (BER)	Repair of damaged bases or single-strand DNA breaks	None reported	None reported	None reported
Mismatch repair (MMR)	Repair of mispaired nucleotides	Mutation of <i>MSH2</i> , <i>MSH6</i> , and <i>MLH1</i> in Turcot syndrome (brain and colon tumors) and <i>HNPCC</i> (colon and gynecologic cancers)	Loss of expression of <i>MSH2</i> or <i>MLH1</i> in sporadic colon cancer	Resistance to DNA monoadducts Sensitivity to DNA cross-links
Nucleotide excision repair (NER)	Excision of a variety of helix-distorting DNA lesions	Mutation of <i>XPA</i> , <i>XPB</i> , <i>XPC</i> , <i>XPE</i> , <i>XPF</i> , or <i>XPG</i> in xeroderma pigmentosum (skin cancer) Variant expression of <i>ERCC1</i> or <i>XPD</i> in lung cancer	Loss of <i>XPA</i> expression in testicular germ cell tumors	Sensitivity to DNA adducts
Homologous recombination (HR)	Repair of double-strand DNA breaks	<i>BRCA1/2</i> mutated in early-onset breast/ovarian, prostate, pancreas, and gastric cancers <i>FANCD1</i> genes mutated in Fanconi anemia	Loss of expression of <i>BRCA1/2</i> in lung, ovarian, and lung cancers Loss of <i>NBS1</i> expression in prostate cancer	Sensitivity to DNA double-strand breaks
Non-homologous end joining (NHEJ)	Repair of double-strand DNA breaks	DNA ligase IV mutated in Lig4 syndrome (leukemia) Artemis mutated in Omenn syndrome (lymphoma)	Loss of Ku70 expression in cervical, rectal, and colon cancers Loss of Ku86 expression in rectal cancer	Sensitivity to DNA double-strand breaks
Translesional synthesis (TLS)	Bypass of DNA adducts during DNA replication	DNA pol E mutated in xeroderma pigmentosum variant (XPV; skin cancers)	Pol β overexpressed in uterus, ovary, prostate, and stomach cancers Pol ι overexpressed in breast cancer	Resistance to DNA adducts

and allows this clamp to interact with the downstream DNA polymerase Pol ϵ . Many of these regulatory processes have been reviewed (21).

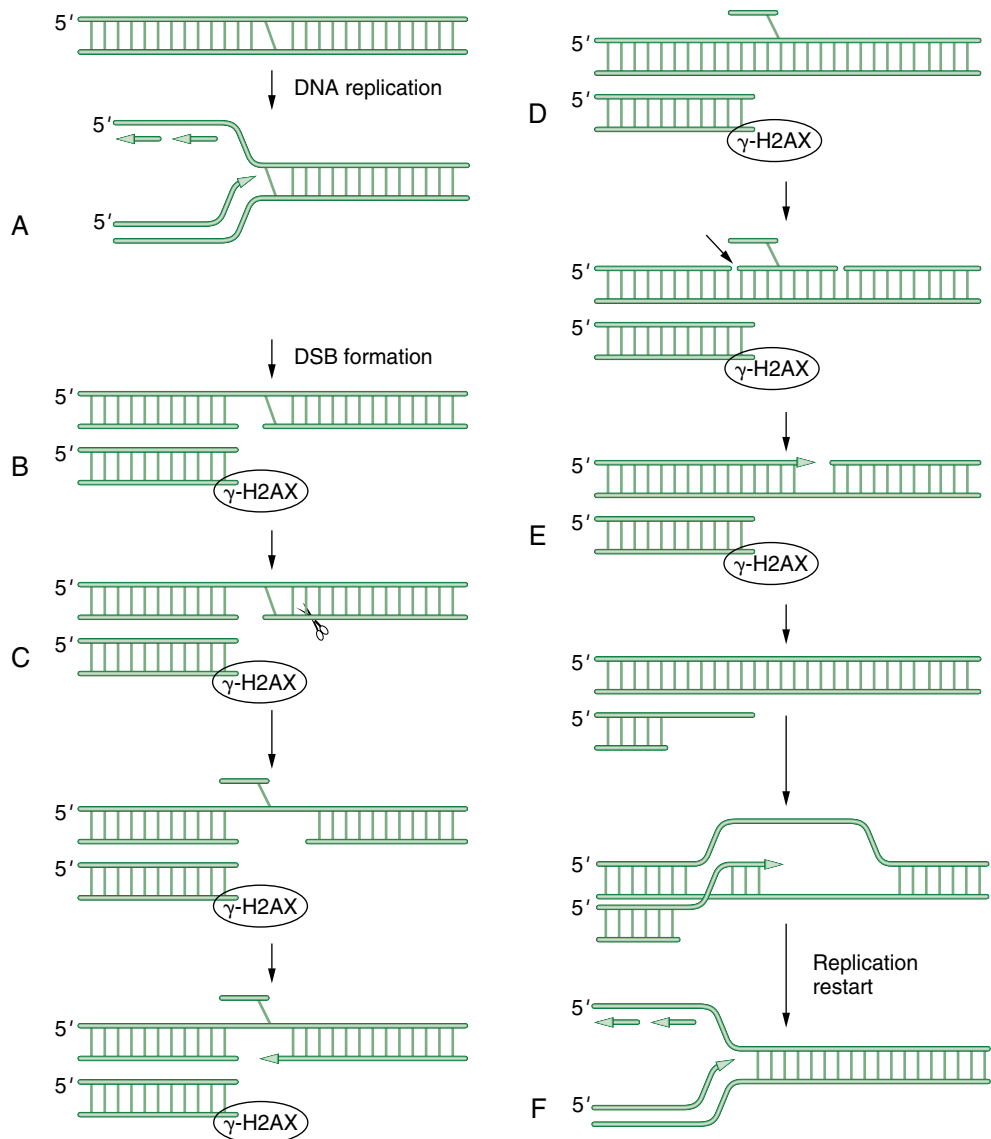
Regulatory enzymes are also required to disassemble DNA repair enzymes after a repair pathway has been completed. For instance, the negative regulatory phosphatase PP2A removes phosphate from ATM substrates and thereby switches off the DNA damage response (22). The deubiquitinating enzyme, USP1, can remove ubiquitin from activated FANCD2 and thereby switch off homologous recombination repair (23). USP1 can also deubiquitinate PCNA and switch off TLS repair (24). These negative regulatory events have also been reviewed (21). The function of these regulatory enzymes underscores the dynamic nature of DNA repair. Loss of these regulatory mechanisms may result in the failure to (1) activate an error-free DNA repair pathway or (2) inactivate an error-prone DNA repair pathway. In either case, the consequence may be a heightened mutation frequency of the dysregulated cell and a predisposition to cancer. Finally, the regulation of DNA repair is a major focus of the DNA repair research field. For instance, it is unknown how DNA repair pathways are activated in specific cell types or at specific stages of the cell cycle. Since some DNA repair processes, such as HR repair, are specifically activated in S phase, it is likely that these pathways are activated by the cdk family of cyclin-dependent kinases.

Sequential Use of Three DNA Repair Pathways to Repair DNA Cross-Links

Interstrand DNA cross-links (ICLs) make up a particular subtype of DNA lesions, and these lesions have an especially potent biologic effect. Because ICLs involve the covalent modification of both strands of DNA, the lesions can prevent DNA strand separation during DNA replication. The lesions can also prevent the access of some DNA repair enzymes and transcription factors that normally require DNA strand separation for DNA binding to occur. DNA cross-linking agents, such as cisplatin derivatives (carboplatin and oxaliplatin) and mitomycin C, are especially cytotoxic to tumor cells, and their therapeutic index derives, at least in part, from the high proliferative rate of tumor cells versus normal cells.

The mechanism of DNA cross-link repair in human cells is poorly understood, and our understanding is derived more from the study of cross-link repair in prokaryotes and in the yeast, *S. cerevisiae*. As shown in Figure 4-7, cross-link repair in human cells probably requires multiple DNA repair pathways. According to this model, the ICL is only repaired during S-phase progression. Initially, an advancing replication fork encounters an ICL. An unknown endonuclease cleaves the DNA, thus generating a DSB. Next, a second endonuclease is invoked to cleave the DNA after the DNA cross-link. This endonuclease may be composed of the ERCC1/XPF proteins.

FIGURE 4-7 A SCHEMATIC MODEL OF DNA CROSS-LINK REPAIR. DNA cross-link repair is believed to occur primarily during the S phase of the cell cycle. When a replication fork encounters an interstrand cross-link (A), DNA replication arrests. Initially, a double-strand break (DSB) is generated by an unknown endonuclease (B). This DSB is next surrounded by phosphorylated Histone γ H2AX. Next, another endonuclease (perhaps ERCC1/XPF) cleaves on the opposite side of the cross-link, allowing extrusion of the cross-linked bases from the double helix (C). Next, a series of three DNA repair pathways act sequentially. Translesion synthesis repair (TLS) allows bypass of the damaged bases (D). Then nucleotide excision repair (NER) excises the damage oligonucleotide and allows gap filling (E). Finally, end resection occurs and the DSB can be repaired by homologous recombination (HR). The replication fork is regenerated in an error-free mechanism (F).



Now that an endonucleolytic event has occurred on each side of the cross-link, the cross-linked single-strand fragment can be flipped out of the helix. This allows three of the normal DNA repair pathways to work sequentially. First, TLS allows bypass of the cross-link and replication and ligation of the upper double helix. Some studies indicate that some variant polymerases, such as Pol η , are particularly important to the TLS across MMC adducts. Next, the NER pathway can excise a stretch of damaged DNA and allow gap filling of the excised oligonucleotide. Finally, HR repair can be used for the error-free, template-driven repair of the damage. The result of this sequential use of three independent DNA repair pathways is to resume DNA replication and restart the replication fork.

Consistent with this model of cross-link repair, some repair-deficient cells are especially prone to the cytotoxic effects of DNA cross-linking drugs. For instance, cells that are deficient in ERCC1/XPF generate the first DSB upstream of the DNA cross-link. However, these DSBs, as measured indirectly by the presence of histone 2AX foci, persist in the repair-deficient cells, suggesting that ERCC1/XPF may work further downstream in the pathway. Similarly, cells deficient in the FA pathway have persistent DSBs after

MMC exposure (25). Thus, the presence or absence of the DSB intermediates is helpful in determining the level at which a repair process is disrupted and the sequence of repair events in the pathway.

DNA Repair and the DNA Damage Response

DNA repair is, in fact, only one class of a broader set of cellular responses referred to as the *DNA damage response*. DNA damage responses include the activation of cell cycle checkpoints, the activation of apoptosis, and the activation of DNA damage tolerance. This latter mechanism allows a cell to “accept” DNA damage and continue DNA replication even in the setting of a heightened mutation frequency. The DNA damage response is therefore a highly coordinated set of signaling events. These responses require a DNA damage sensor (such as a sensor kinase, ATM or ATR), an effector kinase, and downstream protein machines dedicated to DNA repair, apoptosis, or checkpoint activities (26).

DNA Damage Response is Mediated by Sensor and Effector Kinases

The DNA damage response can be activated by a wide range of environmental exposures or drug interactions. An important early player in the damage response is the molecular “sensor” of DNA damage. A local distortion in the DNA double helix, perhaps resulting from a DNA adduct or a thymine dimer, can activate a sensor kinase, such as ATM, ATR, or DNA-PK. These kinases are believed to autophosphorylate (27,28) and go on to phosphorylate a large number of substrates thereafter. The ATM kinase is the product of the ATM gene, the gene mutated in the cancer susceptibility disorder, ataxia-telangiectasia.

Activated ATM and ATR proteins phosphorylate additional downstream “effector” kinases, such as the checkpoint kinases, Chk1 and Chk2. Activated Chk1 and Chk2 then go on to phosphorylate a wide array of protein targets involved in the machinery of DNA repair or DNA damage checkpoints.

One of the best-characterized DNA damage checkpoints is regulated by the ATM-Chk2-Cdc25A axis (29,30). In response to IR, a DSB is generated, and this break activates ATM. ATM subsequently phosphorylates Chk2, which, in turn, phosphorylates the cell cycle activator, cdc25A. Cdc25A phosphorylation leads to its rapid degradation and a cell cycle arrest. This appears to be an important mechanism by which a cell can respond to DNA damage: by arresting its cell cycle progression in S phase. By stopping S-phase entry, a cell allows itself the opportunity to slow down and to repair its DNA or, in the setting of severe damage, to undergo apoptosis. Importantly, a failure to activate this checkpoint response, as in ATM-deficient cells, results in S-phase progression even in the setting of DNA damage. Continuing to replicate DNA in the setting of DNA damage has dire consequences for the cells. The cell may have an elevated mutation rate or may complete DNA replication, only to experience a mitotic catastrophe at the end of the cell cycle.

Failure of ATM to activate the intra-S-phase checkpoint results in a characteristic cellular phenotype. When ATM-deficient cells are exposed to ionizing radiation, they fail to arrest in S phase but instead continue to replicate their DNA and to incorporate tritiated thymidine in the postradiation period. This phenotype is known as radioresistant DNA synthesis (RDS), and it is the hallmark of a cell with a defect in the ATM-Chk2-cdc25A axis. An active area of DNA repair research is the identification of other Chk1 and Chk2 phosphorylated substrates.

Phosphorylated Effector Proteins Assemble in DNA Damage Foci

An important downstream event in the DNA damage response is the assembly of proteins in subnuclear foci (31,32). These foci are often referred to as IRIFs (ionizing radiation inducible foci). Multiple ATM- and ATR-phosphorylated substrates, such as Chk1, BRCA1, and BARD1, assemble in foci following DNA damage. The assembly of these large protein complexes is mediated, at least in part, by the phosphorylated SQ or TQ sequences of the ATM/ATR substrates. Studies have indicated that these

phosphorylated amino acid residues bind directly to phosphoamino acid receptors found on other adaptor proteins. For instance, phosphorylated BACH1 can bind directly to the BRCT domain (a phosphoserine receptor) of the BRCA1 protein (33,34). The precise structure and function of these protein foci in eukaryote nuclei are not known. Clearly, the number of foci correlates with the number of unprocessed double-strand DNA breaks, and the foci are widely believed to be sites of DSB repair.

By immunofluorescence analysis, it is clear that multiple, phosphorylated DNA-damage activated proteins colocalize in these foci. The foci have been helpful to researchers in the establishment of signaling pathways. For instance, pATM, pBRCA1, and pFANCD2 colocalize in IRIFs. Disruption of one upstream protein, say, by a germ line or acquired mutation in the upstream signaling protein, ATM, results in loss of downstream proteins in the foci. Thus, the assembly of the foci has become a useful tool in understanding the interrelationships of DNA-response proteins.

A few DNA damage response proteins deserve special attention here. Bonner and colleagues (31) have identified a variant histone protein, histone 2AX, which is rapidly phosphorylated by ATM after radiation damage. H2AX is an important early-signaling protein in the DNA damage response. The phospho H2AX protein is incorporated in chromatin in vast stretches emanating from the site of the DNA DSB. Absence of Histone 2AX, as in an H2AX knock-out mouse model, results in chromosome instability and cancer predisposition (35,36), apparently due to failure to mount the proper DNA damage response.

Another important DNA damage response protein is RAD51. RAD51 is phosphorylated by the Chk1 kinase during normal S-phase progression (37). RAD51 is a single-strand DNA binding protein that plays a critical role in DNA repair by homologous recombination. Phosphorylated RAD51 also assembles in foci during normal S-phase progression. These “replication foci” are believed to be sites of DNA repair by HR between sister chromatids, which occurs during normal DNA replication.

A comprehensive analysis of proteins that are rapidly phosphorylated after DNA damage (and that form nuclear foci) has provided an important database for laboratories studying the DNA damage response. These phosphorylated proteins, and foci, provide a useful set of biomarkers for DNA repair activities. For instance, cells that are defective in the formation of DNA repair foci are themselves defective in DNA repair. The specific kind of foci that is absent correlates with the particular kind DNA repair deficiency. For instance, cells deficient in RAD51 foci are defective in HR repair and are hypersensitive to IR. Cells defective in the assembly of polyADP ribose (PAR) foci are defective in the repair of single-strand breaks and may therefore have an underlying defect in BER. As such, tumor cells missing particular types of DNA repair foci may be more sensitive to certain kinds of chemotherapy or radiation.

Importantly, human cancers are often deficient in the DNA damage response. Germ-line mutations in DNA damage response genes, such as ATM, NBS1, FANCD2, BRCA1, and BRCA2, can result in an increased susceptibility to cancer. Individuals who inherit a single mutant allele of, for example, BRCA1, have a high risk of developing a breast, ovarian, or prostate cancer during

their lifetime. The tumor results from the inactivation of the second *BRCA1* allele through deletion and loss of heterozygosity, thus resulting in a tumor with a specific DNA repair defect. *BRCA* (–/–) tumors therefore have genomic instability, but also have increased sensitivity to some DNA-damaging agents such as ionizing radiation and DNA cross-linkers.

Study of the DNA damage response reveals that cells have highly regulated responses to different levels and types of DNA damage. Although some DNA repair pathways may be viewed as constitutive, housekeeping pathways, other pathways are highly controlled. Some DNA repair pathways are activated primarily at the site of the advancing replication fork. For instance, ATR and CHK1 are activated at the advancing replication fork, leading to the activation of HR repair (38). Other DNA repair processes are activated in nondividing cells, such as in postmitotic neurons. For instance, NHEJ is hyperactive in nondividing cells and functions as the major mechanism of DSB repair in these cells.

The cellular context of the DNA repair pathway is also important. Germ-line or somatic disruption of a pathway may result in a strikingly different phenotype, depending on the cell and tissue of origin. For instance, gene-line disruption of a DNA damage response, as in the inherited disease, ataxia-telangiectasia, may lead to a characteristic constellation of clinical findings, including cerebella degeneration and lymphoma predisposition. A somatic disruption of the same pathway (e.g., the ATM CHK2-p53) may lead to a very different set of cancers, such as the solid tumors of bladder and ovary (39,40).

Studies indicate that the DNA damage response provides an important “barrier” to the transformation of a normal cell to a malignant cell (39,41). Specifically, early premalignant cells have heightened constitutive activation of the DNA damage response pathways, as exemplified by increased immunohistochemical staining with antibodies to activated ATM and to the activated checkpoint kinase, CHK2. Interestingly, as cells progress from the premalignant state to the malignant state, they lose these DNA damage responses, perhaps through acquired disruptions of ATM or CHK2 activity. Because individuals with genetic diseases, such as ataxia-telangiectasia, already have a defect in the checkpoint response, they may be prone to earlier onset of cancers for this reason.

Inherited Chromosome Instability Syndromes as Models for DNA Repair Defects

Rare pediatric chromosome instability disorders, such as Fanconi anemia and xeroderma pigmentosum, provide important insights to the function of DNA repair pathways and to their role in cancers in the general population. Children born with these syndromes generally have congenital abnormalities, cellular hypersensitivity to DNA-damaging agents, genomic instability, and an increased risk of specific cancers. Although these syndromes are rare, the DNA repair pathways disrupted by germ-line mutations in these individuals are often the same pathways disrupted by somatic mutation

or epigenetic inactivation in cancers from the general population. For these sporadic cancers, a knowledge of which DNA repair mechanism is disrupted provides important clues to the behavior of the cancer or its drug sensitivity spectrum.

At least five of the major DNA repair pathways have corresponding inherited human diseases (Table 4-1). HR and TLS repair is defective in Fanconi anemia cells (42). NER repair is defective in xeroderma pigmentosum cells, Cockayne syndrome cells, and trichothiodystrophy cells (43). MMR repair is defective in children with Turcot syndrome and in tumor cells derived from adult patients with hereditary nonpolyposis colorectal cancer (HNPCC). TLS repair is defective in patients with XP-variant disease. Most of these pediatric diseases exhibit autosomal recessive inheritance, such as XP, Fanconi anemia (FA), and Cockayne syndrome (CS). Turcot syndrome has been reported to exhibit autosomal dominant or autosomal recessive inheritance depending on the particular mutation affecting MMR. Inherited mutations in BER genes have not been observed in humans, suggesting that this pathway is essential for human development.

It is interesting that patients with inherited DNA repair syndromes, such as CS and FA have congenital abnormalities. For instance, CS patients have development abnormalities of the skin and skeletal system. FA patients have skeletal, kidney, cardiac, and bone marrow defects. Consistent with these findings, the NER and FA pathways appear to play dual roles. For instance, the NER excision repair complex, TFIIH, plays an important transcriptional role during embryonic development. Germ-line dysfunction therefore leads to defects during embryonic organogenesis. The NER complex also plays a critical role in DNA repair in somatic cells after organism development. Similarly, the FA pathway appears to have a dual role in development and DNA repair in somatic cells.

The systematic study of these rare diseases has led to a better understanding of (1) the genes and proteins involved in the six major DNA repair pathways; (2) how an inherited (or germ-line) defect in a DNA repair pathway can lead to genomic instability, cancer progression, and drug hypersensitivity; and (3) how an acquired (or somatic) defect in a DNA repair pathway can influence tumor progression and drug sensitivity of tumors in the general population. Although the specific details of these individual inherited diseases is beyond the scope of this review, an example of how a study of these rare diseases can lead to general insights to tumor biology can be appreciated from recent insights into the Fanconi anemia pathway.

Fanconi Anemia: A Specific Inherited DNA Repair Defect

FA is an autosomal recessive or X-linked recessive cancer susceptibility syndrome characterized by multiple congenital abnormalities, progressive bone marrow failure, and cellular hypersensitivity to DNA cross-linking agents, such as cisplatin and mitomycin C (MMC). Patients with FA are prone to developing acute myeloid leukemia as well as squamous cell carcinomas of the head and neck or gynecologic system (44).

The study of FA cells has led to the elucidation of a DNA repair pathway for interstrand cross-links. Clinically, this pathway

is particularly important as many DNA cross-linking agents such as cisplatin, or MMC are used for cancer treatment. The FA defect results from biallelic mutation of any one of 12 known FA genes (A, B, C, D1, D2, E, F, G, I, J, L, M). The proteins encoded by these FA genes cooperate in a common DNA repair pathway, referred to as the FA/BRCA pathway (44). A central event in this pathway is the monoubiquitination of the FANCD2 protein, and this event is a useful biomarker for DNA repair activity (see following section). Disruption of this pathway results in the characteristic clinical and cellular phenotype of FA patients.

Patients with an Inherited Germ-Line DNA Repair Deficiency Exhibit a Characteristic Tumor Spectrum

Patients with inherited DNA repair deficiency syndromes are prone to the development of specific tumors. Patients with FA, for example, are predisposed to acute myeloid leukemia and squamous cell carcinomas, primarily of the head and neck or gynecologic system. Patients with XP are prone to skin squamous cell carcinomas, primarily on body surfaces with more sunlight exposure. Patients with HNPCC and an inherited MMR deficiency are prone to colon cancer and ovarian cancer.

Tumors arising from somatic disruptions of DNA repair pathways may arise in other organ systems. A specific oncogenic lesion, such as the activation of an oncogene or the disruption of a tumor suppressor gene, may have a vastly different effect, depending on the cellular context of the lesion. For instance a germ-line mutation in the retinoblastoma (Rb) gene may result in an embryonal tumor, such as a retinoblastoma or a pineoblastoma, but a somatic disruption of the Rb gene may lead to the development to of a sarcoma.

Similarly, disruptions of a DNA repair pathway, by a germ-line mechanism versus a somatic mechanism, may yield a very different spectrum and behavior. Table 4-1 shows examples. Somatic disruption of the FA pathway results in a wide range of tumor types, including tumors of the ovary, lung, and cervix (42,45). Moreover, somatic disruptions result from methylation and silencing of an upstream FA gene (*FANCF*). Germ-line disruption of the same genes results from inherited mutations, such as missense mutations or nonsense mutations. Somatic disruption of the NER pathway plays a role in the development of testicular cancer and appears to account for the hypersensitivity of this tumor to the drug, cisplatin. Paradoxically, somatic disruption of a DNA repair pathway can also result in chemotherapy resistance. Studies indicate that methylation and silencing of the *MLH1* gene may account, at least in part, for the cisplatin resistance of some ovarian tumors.

Disruptions of the other DNA repair pathways have been observed in sporadic human tumors, accounting, at least in part, for the specific drug- and radiation-sensitivity spectrum of these tumors and their clinical outcome. HR is disrupted in breast and ovarian cancer, NER is disrupted in testicular cancer, and MMR is disrupted in sporadic colon cancer. A few studies suggest that TLS may be disrupted in human cancers. Human cancer cells exhibit an elevation in spontaneous and damage-inducible point mutagenesis compared with nonmalignant cells, suggesting an underlying TLS

defect. An elevation in the expression and activity of the error-prone polymerase, Pol η , accounts for the increase in cisplatin resistance and mutagenesis of these cancers. Consistent with this hypothesis, inhibition of Pol- β in these cells results in resensitization to cisplatin (46).

Somatic Disruption of DNA Repair Pathways by Methylation and Gene Silencing

One of the most common mechanisms of inactivation of DNA repair pathways in sporadic cancer is the epigenetic silencing of a critical gene through methylation of the promoter region. Increasing evidence shows that the FA/BRCA pathway is one of the DNA repair mechanisms that is targeted in sporadic cancers. *FANCF* methylation occurs in 24% of ovarian granulosa cell tumors, 30% of cervical cancer, 14% of squamous cell head and neck cancers, 6.7% of germ cell tumors of testis and 15% of non-small cell lung cancers where it correlates with a worse prognosis. An example of how methylation of a DNA repair gene can promote tumor progression is shown in Figure 4-8.

By regulating the activity of DNA repair pathways, cancer cells have a propensity to progress to a more malignant state. According to this model, early in the course of tumorigenesis, a premalignant cell may undergo methylation and silencing of a DNA repair gene. In the case of the FA/BRCA pathway, the gene most commonly silenced by methylation is the *FANCF* gene on chromosome 11p15. Inactivation of *FANCF* results in a disruption of DNA repair and in genomic instability. The premalignant cell is therefore prone to multiple oncogenic events, such as the up-regulation of a tyrosine kinase oncogene or the disruption of *p53*. A tumor with multiple somatic mutations eventually develops (Figure 4-8), but this tumor still has a defective DNA repair pathway and is hypersensitive to genotoxic chemotherapy. After antitumor therapy, however, there is a selective pressure for tumor cells with an intact FA/BRCA pathway. Tumor cells with a demethylated *FANCF* gene are selected, and a drug-resistant tumor emerges. By following this pattern, tumors can silence and reactivate DNA repair pathways, leading to drug resistance and tumor progression.

The converse scenario may occur for the MMR pathway. MMR-proficient cells are hypersensitive to the DNA cross-linking drug, cisplatin. In this case, it is believed that the active MMR pathway generates a cisplatin-inducible lesion that is tumoricidal. Inactivation of MMR, by methylation of the *MSH2* gene provides the tumor with a mechanism for achieving cisplatin resistance. On the basis of these examples, it would appear that understanding the methylation state of different DNA repair genes may allow the prediction of drug responsiveness of some tumors.

Epigenetic silencing of *BRCA1* through methylation occurs in 13% of breast cancers, 23% of advanced ovarian cancers, 6% of cervical cancers, and 4% of non-small cell lung cancers. Epigenetic disruption of the FA pathway may also be important in the development of sporadic acute myeloid leukemia (AML) where absent

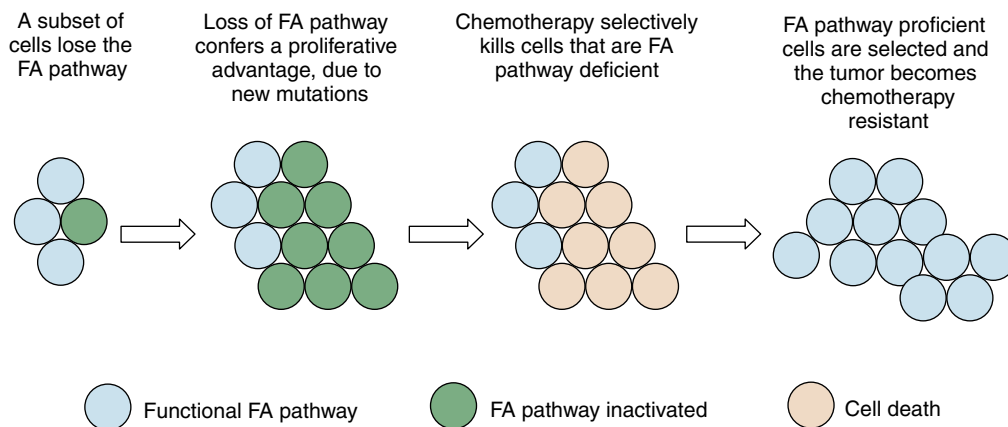


FIGURE 4-8 TUMOR PROGRESSION BY SERIAL INACTIVATION AND REACTIVATION OF DNA REPAIR PATHWAYS. According to this model, early in the course of carcinogenesis, a DNA repair pathway becomes inactivated. For instance, the *FANCF* gene may undergo biallelic methylation and silencing. This loss of the FA/BRCA pathway results in a state of chromosome instability, leading to secondary mutations (activation of K-Ras, inactivation of *p53*, for example). A tumor evolves, and the tumor is initially hypersensitive to cisplatin, as is often the case for ovarian epithelial cancer. Cisplatin causes rapid cytotoxicity of the tumor; however, rare tumor cells undergo a restoration of *FANCF* expression. Restoration may result from an active demethylation of the *FANCF* gene or from positive selection of rare cells that experienced a stochastic demethylation event. Tumor cells regrow and these cells are cisplatin-resistant. In principle, an inhibitor of the FA/BRCA pathway can resensitize the tumor cells to cisplatin, as described in the text.

or reduced expression of the FA proteins FANCA, FANCC, FANCF, and FANCG have been reported. Loss of *BRCA2* mRNA and protein expression has been reported in 13% of ovarian adenocarcinomas; in contrast to the other FA genes described in the preceding paragraphs, this loss does not result from promoter methylation.

Prognostic and Predictive DNA Repair Biomarkers in Cancer Treatment

Hereditary cancer syndromes and sporadic cancers can arise from abnormalities in DNA repair pathways. Clinically, this may be important as these tumors are expected to be hypersensitive to DNA-damaging therapeutic agents or strategies that inhibit alternative DNA repair pathways. In the case of the sporadic cancers, the patient's normal cells, such as those in the bone marrow, possess a functional DNA repair pathway and are predicted to be resistant to these targeted treatments. Assessment of the status of the FA pathway or other DNA repair pathways requires the use of diagnostic biomarkers.

Selection of Biomarkers of DNA Repair Pathways

DNA repair biomarkers of DNA repair pathways can be divided into two major groups: functional biomarkers, which characterize the activity of a pathway after damage, and expression biomarkers, which measure the availability of pathway components prior to damage.

Functional DNA Repair Biomarkers

Functional DNA repair biomarkers indicate an intact DNA repair pathway. These biomarkers have the advantage of giving a functional measure of a particular pathway and will detect repair defects due

to epigenetic events or gene mutations. Moreover, they give a global measurement of a particular pathway's function without needing to know the identities of all the components, some of which may remain unknown. They could also be used to differentiate between insignificant single-nucleotide polymorphisms (SNPs) and functionally important point mutations in DNA repair pathway genes. Functional biomarkers can be applied to serial tumor samples from the same patient at diagnosis and at the time of relapse. In this way, one can determine whether the tumor remains drug sensitive or has restored its DNA repair mechanisms. However these markers rely on tumor tissue having been exposed to some form of DNA damage *in vivo* or *in vitro* prior to the assay. Functional biomarkers of DNA repair pathways include the monoubiquitination of the FANCD2 protein (a biomarker for HR repair) and the phosphorylation of DNA-PK (a biomarker of a functional NHEJ pathway). Abnormal DNA damage-induced nuclear foci may identify disruption of the downstream events in the pathway, such as that observed in *BRCA1*- or *BRCA2*-deficient cells.

DNA Repair Biomarkers of Gene/Protein Expression

DNA repair biomarkers of gene/protein expression indicate the preexisting function of a DNA damage pathway prior to damage. Examples are real-time-polymerase chain reaction (RT-PCR) or immunohistochemistry to test for epigenetic silencing of critical DNA repair genes. Some studies have used a microarray approach to look for genetic expression profiles indicative of abnormal DNA repair gene function. Since some DNA repair genes, such as *MLH1* and *MSH2*, undergo inactivation by methylation, the measurement of gene methylation, using the methylation-PCR assay, can also be applied as a biomarker assay. These approaches have the advantage of not requiring prior DNA damage and can be performed on fixed specimens. However, these assays provide only an indirect measurement of the functional capabilities of a DNA repair pathway. In addition, mutant genes can express normal levels of mRNA and mutant protein and would not be detected by this method.

Clinical Application of DNA Repair Biomarkers

DNA Repair Biomarkers as Predictors of Response to Conventional Therapy

Loss or increased activity of particular DNA repair pathways may influence the response to DNA-damaging therapeutic strategies. For instance, a failure of a pathway involved in the repair of DNA cross-links such as homologous recombination would be predicted to sensitize a tumor to DNA cross-linking agents such as alkylating chemotherapeutic drugs. Indeed, *BRCA1* expression levels as measured by RT-PCR have been used as a biomarker of survival following cisplatin-based chemotherapy for non-small cell lung cancer. Methylation-specific PCR, which indicates loss of gene expression through promoter methylation, has been used to correlate loss of *BRCA1* function with cisplatin sensitivity in ovarian cancer. Loss of *BRCA2/FANCD1* function through mutation in breast or ovarian cancer has also been reported to correlate with a high response to DNA-damaging chemotherapeutic agents. Absence of *FANCD2* monoubiquitination may be a biomarker for loss of function of upstream FA pathway components and could be expected to predict sensitivity to DNA cross-linkers such as cisplatin or cyclophosphamide.

An example of the use of a DNA repair biomarker in clinical medicine is the evaluation of ERCC1 protein expression levels in lung cancer. The NER pathway is important for the correction of UV light-induced thymine dimers and for the excision of small, single-base adducts. In addition, two of the proteins involved in NER, ERCC1 and XPF, appear to have special relevance to the repair of DNA interstrand cross-links. Primary cells derived from XP patients with germ-line mutations in ERCC1 or XPF are hypersensitive to UV and DNA cross-linking agents (47). The protein level of ERCC1 in cell lines correlates with the level of functional DNA cross-link repair in the cell.

One group performed a retrospective analysis of 700 patients with non-small cell lung cancer who had been treated with adjuvant chemotherapy, including cisplatin(66). The banked primary tumor samples, which were stored in paraffin blocks, were evaluated for the level of ERCC1 protein using immunohistochemistry (IHC). Interestingly, the patients with tumors exhibiting low ERCC1

levels were more sensitive to cisplatin, based on their longer average time to relapse after cisplatin, compared with patients whose tumors had high levels of ERCC1. The results of this study indicate that ERCC1 protein expression may be a useful predictive biomarker for assessing tumor response to cisplatin.

DNA Repair Biomarkers to Guide Chemo- and Radiosensitization

Resistance to DNA-damaging chemotherapy or radiotherapy may be due to enhanced repair of DNA lesions. Therefore, a possible therapeutic strategy is to use drugs that specifically inhibit DNA repair pathways. Theoretically, this strategy may be limited since the drug may also increase the toxicity of therapeutic DNA damage in normal tissue. A therapeutic index can be achieved, in principle, by (1) the selective uptake of the DNA-damage sensitizers by the tumor cell versus the normal cell or (2) by delivering one of the modalities (such as the radiation) directly to the tumor.

An understanding of the precise molecular mechanisms of new classes of sensitizing agents has important implications. First, if an agent functions by inhibiting a specific DNA repair pathway, active derivatives of this agent should function similarly. DNA repair pathway inhibition provides an important biomarker for determining the proper dosing of the drug. Second, the chemosensitizer would be predicted to be more efficacious when used in combination with specific classes of DNA damage drugs.

DNA Repair Biomarkers as Predictors of Response to Targeted Monotherapy

Another important application for biomarkers of DNA repair pathway integrity is the potential to develop nontoxic monotherapy for tumors with specific DNA repair defects. The up-regulated DNA repair pathway is the "Achilles heel" of the cancer. In principle, a nontoxic inhibitor of this second pathway, delivered as a monotherapy, may selectively kill the cancer cell. A normal cell, in comparison, may be able to tolerate the loss of this second pathway since other pathways are functioning, and there is more redundancy in its DNA repair capacity. The principle of monotherapy for cancer cells with a defect in DNA repair is shown in Figure 4-9.

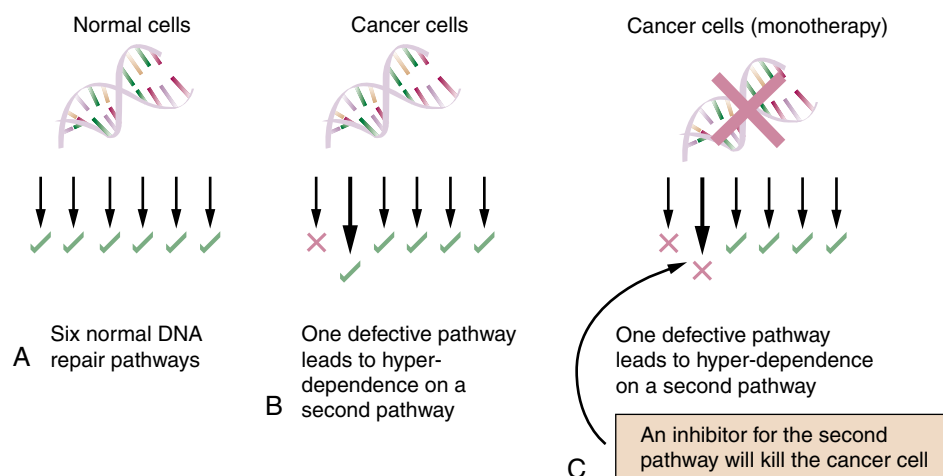


FIGURE 4-9 PRINCIPLE OF DNA INHIBITOR MONOTHERAPY. **A:** Normal human cells have six DNA repair pathways. **B:** Tumor cells, in contrast, have disrupted one DNA repair pathway, through somatic mutation, loss of heterozygosity (LOH), or epigenetic silencing of a DNA repair gene in that pathway. The tumor cell has genomic instability and has partially compensated for its DNA repair defect by up-regulating a second pathway. For instance, breast tumors often have a defect in homologous recombination (HR), and these tumors up-regulate base excision repair (BER) for their survival. **C:** The tumor cell is hyperdependent on this second pathway, and a specific inhibitor kills the tumor cells but has little effect on the normal cells. An example of this monotherapy approach has recently been described for PARP inhibitors.

This principle has been demonstrated by the use of PARP inhibitors in *BRCA1*- and *BRCA2*-deficient cells (11,12). As discussed earlier, under normal physiologic conditions, DNA is damaged continuously. The result of these stresses is the development of damaged bases or regions of single-strand DNA breaks (SSBs), which are repaired through the BER pathway. Part of the BER pathway requires polyADP ribose polymerase (PARP), a DNA-binding zinc finger protein that catalyzes the transfer of ADP-ribose residues from NAD⁺ to itself and different chromatin constituents, forming branched ADP-ribose polymers. Initially it was observed that PARP-deficient (and therefore BER-deficient) mice develop normally but have high levels of sister chromatid exchange, a feature of HR. This observation suggested that HR could compensate for a loss of PARP-dependent BER. Consequently it was demonstrated in preclinical models that *BRCA1/BRCA2*-deficient human and murine cells were sensitive to PARP-inhibiting drugs, whereas cells expressing normal levels of *BRCA1* or *BRCA2* were unaffected. PARP1 inhibitors are well tolerated in preclinical murine models and in addition to being a potential treatment for *BRCA1/BRCA2*-mutant tumors, may also represent an attractive strategy for chemoprevention of malignancies in mutation carriers. Clearly a biomarker that indicates a failure of *BRCA1/BRCA2* function in tumor cells may allow the application of PARP inhibitors to a wider spectrum of sporadic human malignancies

Development of new DNA Repair Biomarkers

Few biomarkers exist for evaluating the integrity of the other DNA repair pathways. Several studies have attempted to assay these pathways, using expression biomarkers (i.e., the testing the expression levels of known DNA repair proteins in the pathways). Better functional biomarkers are needed. Some studies have indicated that post-translational modifications of DNA repair proteins in these pathways are also required for pathway activity. For instance, polyubiquitination of XP-C is required for functional NER (20), and sumoylation of thymine-DNA glycosylase (TGD; 18) is required for function of BER. The development of antibodies specific for these activated states and the testing of these biomarkers may allow the rapid assessment of drug sensitivity and acquired resistance in clinical samples.

DNA Repair Inhibitors as a New Area for Anticancer Drug Development

As shown in Figure 4-9, normal human cells may have six functional DNA repair pathways, while a tumor cell may have disruptions of one pathway. In the tumor, disruption of one pathway, such as HR repair, results in genomic instability and hyperdependence on a second pathway, such as BER. Because of this hyperdependent state, the tumor cell may be hypersensitive to an inhibitor of BER, such as a PARP1 inhibitor.

In this case, the tumor may respond to the PARP1 inhibitor as a single agent (monotherapy; 48–50). DNA damage, activated by the hyperproliferative state of the tumor cell, may be sufficient to kill the tumor cell. Alternatively, the PARP1 inhibitor may have a greater tumoricidal effect when it is used in combination with another cytotoxic agent, such as IR or an alkylating agent Temozolomide (TMZ).

Based on the early success with PARP1 inhibitor therapy, there is increasing interest in the identification of inhibitors of other DNA repair pathways (51). For instance, an inhibitor of the sensor kinase, ATM, has been shown to have potent tumoricidal effects (52). Also, inhibitors of the Chk1 kinase, UCN01, have been in clinical trials (26,53). Investigators have also begun to screen for inhibitors of homologous recombination repair that may potentially sensitize tumor cells to IR or to cross-linker damage (54). While DNA repair inhibitors may sensitize a tumor to the cytotoxic activity of conventional IR or chemotherapy, they may also enhance the toxicity of these therapies to normal human cells.

Importance of DNA Repair to Clinical Oncology: Other Specific Examples

BRCA1 and *BRCA2*

The importance of DNA repair to the pathogenesis and treatment of cancer is exemplified by studies of the breast cancer susceptibility gene, *BRCA1*. Approximately 10% of women who develop breast cancer in their lifetime have a strong family history of (inherited) breast cancer. Of these women, approximately half are heterozygous carriers for mutations in either the *BRCA1* or *BRCA2* gene.

The *BRCA1* gene was originally mapped to human chromosome 17 (55), and it was subsequently cloned by position (56). Strong evidence emerged that *BRCA1* is a tumor-suppressor gene, since breast carriers have loss of heterozygosity (LOH) at the *BRCA1* locus (57). *BRCA1*-deficient breast tumor cells are hypersensitive to IR and to DNA cross-linking agents, suggesting that *BRCA1* may function in the regulation of homologous recombination repair.

Studies with the *BRCA1* protein indicate that, during the DNA damage response following cellular exposure to a genotoxic stress, *BRCA1* is phosphorylated and accumulates in subnuclear foci THAT colocalize with *BRCA2* and *RAD51* proteins (58,59). These foci are required for competent DNA repair. The precise role of *BRCA1* in DNA repair is unknown. Since *BRCA1* is itself an E3 ubiquitin ligase (60), it may function by ubiquitinating other DNA repair proteins and regulating DNA repair indirectly. Some studies have identified key ubiquitinated substrates of *BRCA1* including the protein, CTIP (61).

Since *BRCA1* (and *BRCA2*) tumors are deficient in HR repair, this genotype may be useful in the selection of the chemotherapy. Studies indicate that *BRCA1*-deficient tumors are hyperdependent on BER and have elevated PARP1 activity (11,12). Accordingly, *BRCA1*- and *BRCA2*-deficient tumors appear to be hypersensitive to PARP1 inhibitors.

Defects in DNA Repair Pathways can Account for Elevated Mutation Rate of Cancer

Cancer cells have an increased mutation rate compared with normal cells, and this phenotype has important clinical consequences. The increased mutation frequency can lead to point mutation and inactivation of tumor suppressor genes or to increased tumor cell resistance to chemotherapy. The increased mutation rate may also account for the increased spontaneous cell death observed in solid tumor samples (i.e., some of the mutations may be lethal to individual tumor cells), but may also enhance the outgrowth of a more malignant clone.

This increased mutation rate results in large part through the disruption of DNA repair pathways. The MMR pathway normally functions to improve the fidelity of DNA replication by quickly identifying and excising mismatched bases generated by faulty DNA replication. Loss of the MMR pathway by germ-line mutation or somatic mutation, can lead to a “mutator” phenotype. This phenotype can be readily detected by microsatellite instability in the genome of the cancer cell.

This increase in mutation rate can also be accounted for by an increase in error-prone DNA repair mechanisms (62). In the setting of elevated translesion synthesis, some error-prone polymerases, such as Rev3, may increase the frequency of point mutations in the genome of the human cancer. Also, an elevation in the error-prone NHEJ pathway may account for the elevated complex mutations (insertions and deletions), observed in some cancers. Many human tumors have been found to express abnormal levels of polymerase β (63,64), which may also contribute to their increased mutation frequency.

Multiple Mechanisms of Cisplatin Resistance

Studies indicate that the status of DNA repair pathways in human tumors may be highly predictive of cisplatin sensitivity. As mentioned previously, defects in the NER pathway may account for cisplatin sensitivity of some testicular and non-small cell lung cancers.

Defects in HR repair may account for cisplatin sensitivity of ovarian and head and neck carcinomas. Other cellular mechanisms may also account for the intrinsic cisplatin resistance of many human tumors. Cisplatin-mediated tumoricidal activity can be affected by (1) the expression of cell surface P-glycoprotein, an efflux mechanism for removing cisplatin; and (2) the relative anti-apoptotic state of the tumor cell based, at least in part, on the level of BCL-2 and BCL-X expression. Primary cisplatin resistance may therefore rely on the systematic assay of many of these mechanisms in a given tumor cell (65).

DNA Repair Gene Polymorphisms as Predictors of Chemotherapy Responsiveness

As described previously, disruption of the NER pathway appears to account, at least in part, for the cisplatin hypersensitivity of

testicular cancers and of some ERCC1-deficient non-small cell lung cancers (66). The disruption of the NER pathway may result from definitive mutations (i.e., frame shift or nonsense mutations) in NER genes or from epigenetic changes, such as methylation and silencing of NER genes. In some cases, the disruption of the NER pathway may be partial, and it may result from DNA repair gene polymorphisms carried in the germ-line of the cancer patient.

In principle, DNA repair polymorphisms may result in a more subtle DNA repair defect. Such a defect may increase the risk that an individual develops a cancer or may increase the likelihood that the resulting tumor is sensitive to a specific genotoxic agent. Based on this idea, investigators have screened large tumor sets for the enrichment of particular SNPs in DNA repair genes. Common SNPs are known for the NER genes *XPD*, *ERCC1*, and *XRCC1*. SNPs in multiple NER genes appear to account, at least in part, for the cisplatin hypersensitivity of some squamous cell carcinomas and lung cancers. Whether these SNPs will serve as predictive biomarkers for chemotherapy or radiation sensitivity remains unproven.

Conclusion

Genomic instability is characteristic of most human malignancies, and this phenotype can arise from acquired defects in any one of six DNA repair pathways. These pathways are MMR, BER, NHEJ, NER, HR, and TLS. The germ-line disruption of these pathways accounts for the pathogenesis of several inherited DNA repair disorders including FA, XP, and HNPCC. The somatic disruption of these pathways can account for the genomic instability and drug sensitivity of many tumor types. The six pathways differ significantly in their ability to repair modified DNA bases, DNA cross-links. Different cell types and tumor cell types have differential dependence on these pathways for growth and survival.

In the future, the development of biomarkers for the function of other DNA repair pathways may allow better targeting of conventional agents or the use of monotherapies designed to inhibit specific repair pathways. The biomarkers can also be used as screening tools to find inhibitors of DNA repair that function as chemosensitizers. We predict that these approaches should reduce the toxicity of existing cancer treatments by eliminating the use of noneffective agents and by directing the development of novel treatment strategies.

Understanding the status of DNA repair pathways in tumor cells will have considerable use in clinical oncology. If a tumor is defective in one pathway (e.g., NHEJ), it may be directly sensitive to IR, a modality that generates double-strand breaks in DNA. If a tumor is defective in another pathway (e.g., HR) it may be hyperdependent on a second pathway (e.g., BER) for its survival. Accordingly, a drug, such as a PARP1 inhibitor, which targets the BER pathway, may be selectively toxic to these tumors.

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Epigenetics and Cancer

It has been known for decades that genetic alterations are a fundamental driving force in the initiation and progression of human cancers. It is also now apparent, through a more recent body of work, that epigenetic changes may be equally as important in tumor development. Epigenetics refers to heritable changes in gene expression in somatic cells that are determined by other than alterations in the primary base sequence of DNA (1). Normally, it is such epigenetically mediated gene expression profiles that mediate the changes in cell phenotype that must evolve against the background of an individual's uniform DNA sequence during such processes as embryonic development and cell differentiation. Just as DNA mutations can mediate individual stages of tumor development by fostering over-, or under-, function of key genes, epigenetic abnormalities can heritably allow similar gene expression aberrations. In this chapter, features are outlined of this latter form of altered gene function and how it is coming to impact the understanding and management of human cancer.

The Molecular Basis for Epigenetic Control of Gene Expression

While the primary base sequence of DNA obviously specifies for the sequences determining the content of transcribed RNA, and the corresponding amino acid sequence of encoded proteins, DNA sequence cannot, per se, determine which regions of our genomes get expressed. Rather, it is the nuclear packaging of the DNA, and the resultant availability, or exposure, of DNA regions to the transcriptional machinery that determines the gene expression profile of any given cell type (1). In turn, this nuclear packaging is accomplished through a complex and dynamic interaction of DNA with proteins, which constitutes cellular chromatin, the post-translational modifications of these interacting proteins, the positioning of the DNA-protein interfaces, and DNA methylation, a covalent mark constituting the only postreplicative modification made to DNA (1). Alterations to all of these processes are being increasingly identified as important to the evolution of cancer.

The basic molecular unit of DNA packaging is the nucleosome (Figure 5-1), a structure characterized by the wrapping of ≈ 146 bp of DNA around what is termed the "histone octamer,"

consisting of a tetramer of two histone H3, H4 dimers and a dimer of histone H2 (2). Groups of nucleosomes may in turn be organized into higher order structures, through actions of chromatin remodeling complexes (3–5), consisting of tightly compacted aggregates, or "closed chromatin," characteristic of DNA regions that are transcriptionally silent, versus more irregularly, and linearly spaced, nucleosomes, or "open" chromatin, characteristic of DNA regions where transcription is active (6,7).

One of the most exciting and dynamic areas of chromatin biology concerns another key facet of nucleosome function that helps determine the transcriptional status of genomic regions, post-translational modification of key amino acid residues of the histones (Figure 5-2). These modifications constitute what has been termed the "histone code" for gene expression regulation (8–10). Thus, acetylation of key residues, such as lysine 9 of histone H3 (H3K9acetyl), by enzymes known as histone acetylases (HATs) usually specifies for transcriptionally inactive regions while deacetylation at these sites, mediated by histone deacetylases (HDACs), is usually associated with transcriptional repression. Methylation of key amino acids also occurs and may be an activating or inactivating mark, depending on the site. For example, the mark of H3K4 methylation is enriched at active areas while H3K9 methylation or H3K27 methylation is characteristic of transcriptionally repressed regions (8–10). These methylation marks are controlled by a dynamic process involving individual histone methyltransferases, which place the marks, and demethylases, which can remove them (11–15).

Interacting with all of the dynamics for nucleosome assembly, placement, and histone modifications, to mediate nuclear packaging of DNA, is the DNA modification of DNA methylation (Figures 5-1 and 5-2). This process, which in mammalian cells involves methylating the DNA at cytosines that are located 5' to guanines, or at the CpG dinucleotide, is mediated by three DNA methyltransferase enzymes that utilize S-adenosyl-methionine as a methyl donor group to transfer this moiety for covalent linkage to the cytosines (6,7). DNA methylation adds a dimension to packaging of DNA and nucleosomes into repressive domains by stabilizing the heritable nature of transcriptional silencing (6,7). A key aspect of this dimension concerns the distribution of DNA methylation in the genome (Figure 5-1). In most genomic regions, the CpG dinucleotide is underrepresented because, over evolution, these cytosines have been depleted because deamination of

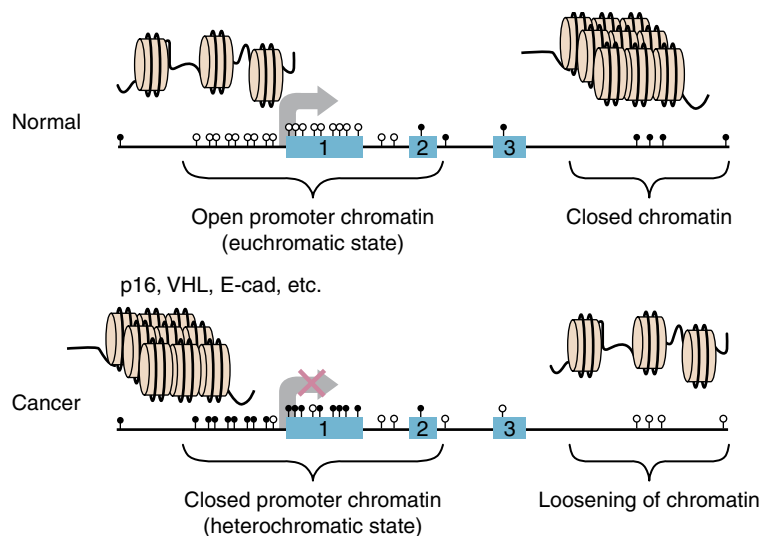


FIGURE 5-1 THE NORMAL VERSUS CANCER EPIGENOME. **Top:** In normal mammalian cells, CpG islands in proximal gene promoter regions (a three-exon gene is shown, with each exon marked in blue and numbered) are largely protected from DNA methylation (cytosines, *open lollipops*) and reside in restricted regions of open chromatin (inset, upstream of transcription start shows three nucleosomes with wide spacing), or euchromatic states, favorable for gene transcription (*large blue arrow*). In contrast, for most regions of the genome, such as in the bodies of many genes and areas outside genes, particularly including repeat elements and pericentromeric regions, the cytosines in CpG dinucleotides are methylated (*black lollipops*). This DNA methylation is characteristic of the bulk of the human genome, which is packaged as closed chromatin (the inset above methylated CpGs shows multiple nucleosomes with higher-order, tight compaction) unfavorable for transcription. **Bottom:** In cancer cells, there tends to be a reversal of this pattern. Proximal promoter CpG islands for many abnormally silenced genes (as represented by the same gene as shown in the top panel, and which is depicted as representing the tumor suppressor genes listed) become DNA hypermethylated and reside in a closed chromatin, or more heterochromatic-type state, which is not favorable for transcription (*red X*). In contrast, cytosines in CpG dinucleotides in other regions of the genome display hypomethylation and are associated with states of aberrantly loosened chromatin. The overall result is abnormal chromatin packaging with the potential for underpinning an abnormal cellular memory for gene expression and for conveying abnormal structural function for chromosomes. (From Ting AH, McGarvey KM, Baylin SB. *The cancer epigenome: components and functional correlates*. *Genes Dev* 2006;20:3215–3231, with permission.)

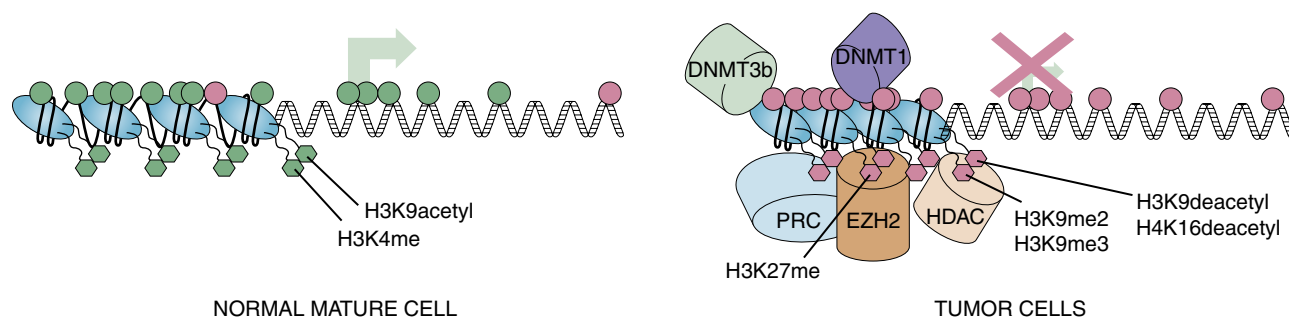


FIGURE 5-2 Chromatin surrounding an actively expressed gene in a normal mature cell versus surrounding that same gene when it is DNA hypermethylated and aberrantly, heritably, silenced in a tumor cell. On the left, the chromatin is composed of histone modifications associated with active transcription (H3K4me) and (H3K9 acetyl) and the DNA is largely unmethylated at CpG sites (*green circles*) with only occasional methylation (*red circles*). The nucleosomes (*large blue ovals*) are linearly arranged as associated with the areas of active transcription defined in [Figure 5-1](#). The gene on the right is fully transcriptionally repressed (*large red X*), the DNA is methylated and DNA methylating enzymes are present (DNMT-1 and -3b), HDACs are present to catalyze histone deacetylation, the machinery of transcriptional repression is present including the PcG proteins (PRC) with EZH2, which catalyzes the H3K27me3 mark (*red hexagons*) and the key silencing marks of H3K9me2 and me3 are also present (*red hexagons*). The nucleosomes are more tightly compacted as is representative of the repressive chromatin shown in [Figure 5-1](#). (From Ting AH, McGarvey KM, Baylin SB. *The cancer epigenome: components and functional correlates*. *Genes Dev* 2006;20:3215–3231, with permission.)

methyl-cytosines leads to replacement with thymines (6,7). However, as many as 80% of these remaining CpG sites are DNA methylated in the human genome and this has an important functional correlate. This methylation corresponds to the fact that most of our genome, in adult cells, is packaged away into nucleosome-compacted DNA characteristic of regions of transcriptional repression ([Figure 5-1](#)). This may constitute one of the most important functions of genomic DNA methylation, which is to ensure tight heritability of overall genomic transcriptional repression to prevent unwanted expression of elements such as viral insertions, repeat elements, and other potentially deleterious sequences (16).

In contradistinction to the depletion of CpGs throughout most of the genome, approximately half of the genes in the genome have regions in their promoters, termed “CpG islands,” where the expected frequency of this nucleotide has been preserved ([Figure 5-1](#)). For most such genes, these islands are protected from DNA methylation, and this methylation-free state is associated with active transcription of these genes, or preservation of their being in a transcription-ready state (6,7,17). These CpG islands are the target of key epigenetic abnormalities in cancer cells as discussed in detail in subsequent sections of this chapter.

In addition to the previously described postulated role of DNA methylation in global DNA packaging, it is also linked to regulation of expression for specific genes in normal cells. In this regard, when localized to gene promoter regions, it may act to provide a tightening of heritable states for gene silencing. Examples include the imposition of DNA methylation in the promoters of genes shortly after other processes initiate their silencing in regions on the inactive X-chromosome of females (18). A similar role is apparent in genes that are imprinted in mammals wherein DNA methylation of promoter regions is seen on the silenced allele of such genes (19,20). DNA methylation also may participate in regulating expression of certain genes in normal cells, which are expressed in a tissue-specific manner, such as the silencing of globin genes in all but cells actively engaged in erythropoiesis (21,22).

In the gene-silencing roles, there is a tight interplay between the modification of key histone amino acid residues and DNA methylation. Thus, at least in lower organisms such as *Neurospora* and *Arabidopsis*, methylation of lysine 9 of histone H3 (H3K9me), may help determine positions where cytosine methylation is placed in the genome (23,24). In turn, DNA methylation recruits a series of proteins, methylcytosine binding proteins (MBDs), which are complexed, in turn, with histone deacetylases (HDACs), which help maintain the deacetylation of H3K9 and other key histone lysines in regions of silenced genes (6,7).

Abnormalities of DNA Methylation and Chromatin Organization in Cancer: the Cancer “Epigenome”

Overall Characterization

The organization of the genome, as mediated by chromatin and DNA methylation, appears to be quite abnormal in cancer cells of all types when compared with the relevant comparative cells in normal renewing adult tissues (25–27). In many cancers, total levels of DNA methylation are decreased with losses apparent within repeat sequences, the bodies and promoters of selected genes, and in the pericentromeric regions of chromosomes (25–27). The full ramifications of these losses are still being explored, but the changes have the potential for associating with unwanted gene expression, and especially, in terms of the pericentromeric abnormalities, with chromosomal instability (25–29).

In the setting of the losses of DNA methylation, more localized gains in gene promoter regions has become the most studied of the epigenetic changes in cancer. These gains, accompanied by a series of the repressive chromatin changes discussed earlier, are associated with an aberrant loss of gene expression (25–27,30). In fact, it is increasingly apparent that disruption of gene function as a consequence of promoter DNA hypermethylation is as frequent, or more frequent, in cancers than mutations as a mechanism for loss of tumor suppressor gene function (25). Individual tumors may actually contain hundreds of such affected genes (31). Genes affected, which involve virtually half of the best characterized tumor suppressor genes (25–27,30), include those involved with virtually every cellular pathway (Table 5-1; 25–27,30,32),

Table 5-1 Examples of Pathways Affected by Aberrant Gene Silencing in Cancer

	Pathway Genes
Cell cycle control	<i>p16, p15</i>
Apoptosis	<i>DAP-kinase, ASC/TMS1, HIC1</i>
Increased stem/developmental pathway activity (Wnt, etc.)	<i>SFRPs</i>
DNA damage repair	<i>MLH1, O⁶-MGM, GST P1</i>
Cell adhesion	<i>E-cadherin</i>
Cell migration	<i>TIMPs</i>
Differentiation	<i>GATA-4, GATA-5, TGF-β receptor</i>
Chromosomal stability	<i>CHFR</i>

which, when disabled, results in fostering initiation and progression of tumors including controls for cell cycle events, apoptosis, developmental biology signal transduction for stem cell function, differentiation, cell–cell adhesion, cell–cell recognition, cell migration and invasion, and so forth (32). The list of involved genes, as identified by study of candidate genes and techniques for randomly screening the cancer epigenome (30,31,33) is steadily growing for virtually all major cancer types.

Interplay between DNA Methylation and Chromatin in Cancer Cells

One of the most active areas of cancer epigenetics research at present, and one of utmost importance to the translational impact for cancer prevention, diagnosis, and treatment, concerns delineation of the molecular underpinnings of how the cancer epigenome evolves—especially how the aberrant promoter DNA methylation and gene silencing are initiated and maintained. This investigation has benefited from, and contributed to, the explosion of knowledge over the past 5 to 10 years in understanding of how chromatin functions for packaging of the genome and for direct modulation of gene expression. While much remains to be elucidated, important findings are merging that provide clues to the origins of epigenetic abnormalities in cancer.

The initiation of DNA methylation, its maintenance, and its role in transcriptional repression are all dependent on its interaction with chromatin organization (Figure 5-2). As previously alluded to, the sites if DNA methylation themselves may be dependent, initially, on histone modifications. Thus, H3K9 methylation, and the histone methyltransferases that catalyze this mark, appears required for DNA methylation in lower organisms such as *Arabidopsis* and *Neurospora* (23,24). In addition, the polycomb group of proteins (35–37), discussed in more detail later, which target another key gene repression mark to nucleosomes, H3K27me, have been implicated in the targeting and maintenance of DNA methylation. In addition, a series of proteins, called methyl cytosine binding proteins (MBPs), and the protein complexes in which they reside, can bind to methylated CpG sites to help relay a silencing signal (6,7). These complexes contain the previously mentioned enzymes, histone deacetylases (HDACs), which catalyze the deacetylation of key amino acid residues, such

as H3K9, that are highly characteristic of transcriptionally silent regions of DNA (6,7,9). The DNMTs themselves also interact with HDACs to help target these enzymes to sites of DNA methylation (38–40).

The alterations in the levels or ratios of factors that mediate epigenetic abnormalities in cancer cells are first manifest by certain global abnormalities. Thus, increases in the levels and activities of the DNA methylation catalyzing enzymes (41), of the proteins in complexes that modulate the enzymes that catalyze transcriptional repression histone modifications (42–44), as well as altered levels of the repressive histone marks themselves, including loss of acetylation at H4K16, and increased levels of H4K20 acetylation (45), are all reported as common hallmarks of human cancer. Locally, at gene promoters affected by promoter DNA methylation and aberrant gene silencing (Figure 5-2), there are decreases in histone modifications associated with active gene transcription, such as acetylation of H3K9 and H4K16, increases in modifications associated with transcriptionally repressive chromatin including H3K9me2 and me3, and H3K27me3, and in the enzymes that catalyze these latter repressive marks (41,46).

The precise manner in which all of these chromatin components interact to initiate and/or maintain abnormal gene promoter DNA methylation and the attendant silencing of involved genes is not yet known. Key clues are coming from studies of human and murine embryonic cells, which suggest, that at least for certain groups of genes, the manner in which chromatin is organized at the gene promoters in stem/precursor cells may make them vulnerable to abnormal DNA methylation during the abnormal cellular expansion that underlies the earliest phases of tumor progression (41,47–49). The possibilities that now arise from the data at hand are summarized in Figure 5-3. A key mark in human embryonic stem cells for many of the genes that become DNA hypermethylated in adult cancers is the PcG-mediated H3K27me3 histone modification (41,47–49). This alone, however does not seem responsible for the DNA methylation since most of these genes in embryonic stem cells or even tumors of such cells, do not generally have this change (49). It appears possible that addition of the H3K9 me2 and me3 marks to the H3K27 me3 mark may be involved since these may precede the appearance of abnormal promoter DNA methylation and are characteristic of genes that are DNA hypermethylated in adult cancers (41,49).

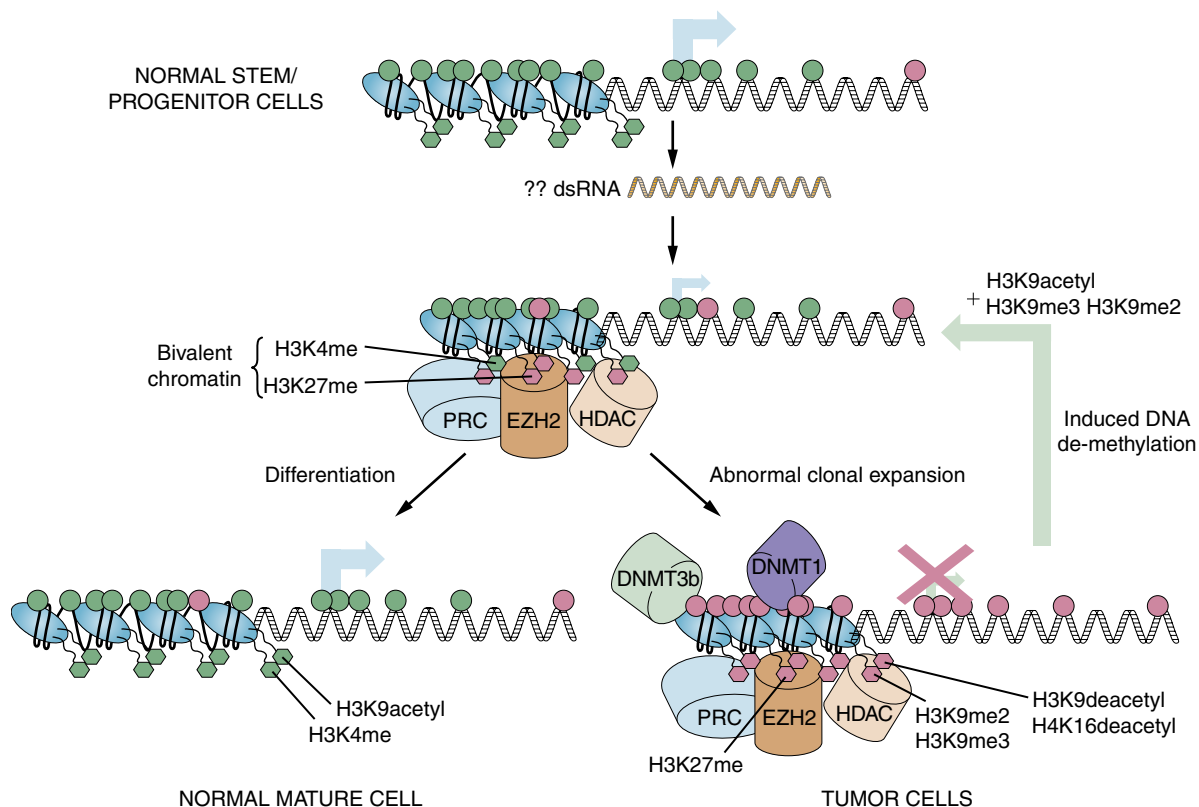


FIGURE 5-3 A MODEL FOR THE POTENTIAL CONTRIBUTION OF STEM CELL CHROMATIN TO THE INITIATION AND MAINTENANCE OF ABERRANT EPIGENETIC GENE SILENCING IN CANCERS. During normal ES cell formation, a bivalent chromatin is recruited to the promoters of a subset of genes that need to be held in a low expression state to prevent lineage commitment. The involvement of small interfering RNA (siRNA) species could be a trigger to this process, and the chromatin is composed of histone modifications associated with active transcription (H3K4me) and inactive transcription (H3K27me). The PRC are responsible for the H3K27me3 mark through the HMT, EZH2, and deacetylation of key histone lysine residues is catalyzed by HDACs that are recruited by multiple transcriptional repressive complexes. At such genes, DNA is largely unmethylated (green circles), and histones may be maintained in a mixture of acetylated (green hexagons) and deacetylated (red hexagons) states. **Bottom left:** With normal cell differentiation and lineage commitment, the genes become transcriptionally active, and the silencing marks are reduced while active histone marks are retained. DNA remains unmethylated. However, as shown in the bottom right, during cancer-predisposing events, abnormal pressure for stem/progenitor cell proliferation with retained bivalent chromatin may allow polycomb proteins and/or marks to recruit other silencing marks such as H3K9me2 and H3K9me3 and DNMTs. The promoter evolves abnormal DNA methylation (red circles) and a tight heritable gene silencing (large red X), which results in loss of function for genes. Tumors may arise in such clones with subsequent progression steps. Experimentally, the potential underlying bivalent chromatin for such tumor genes, plus retained H3K9me3, can be revealed by induced DNA demethylation (large green arrow) and resultant gene re-expression. (From Ting AH, McGarvey KM, Baylin SB. The cancer epigenome: components and functional correlates. *Genes Dev* 2006;20:3215–3231, with permission.)

Relationships of Epigenetic Changes in General, and Aberrant Gene Silencing in Particular, to the Progression of Cancer

While losses and gains of DNA methylation in cancer may arise at any point during tumor progression, it has become apparent that many of the changes arise early, prior to frank carcinomas (30,32,41,50). In fact, it is possible that some of the events, such as silencing of key genes, may initiate the abnormal clonal expansion that creates early preinvasive lesions, which are then at risk for subsequent genetic and epigenetic events that further tumor progression and lead to invasive and metastatic cancer (Figure 5-4; 30,32,41,50). The genes silenced may provide loss of tumor suppressor function that allows cells to abnormally survive the hostile environments that are risk factors for cancer development, such as chronic inflammation, and expose cells to DNA damaging agents such as reactive oxygen species (ROS). Cells involved in injury repair might normally undergo apoptosis from such DNA damage, but if able to survive, and expand, may select for mutations and/or chromatin damage which may favor subsequent tumor progression (Figure 5-4).

There are now several key examples of the early role for DNA hypermethylation and gene silencing in tumor progression. One of the major tumor suppressor genes in cancer, where loss of function leads to cell cycle abnormalities and uncontrolled growth, is *p16* (51). A role for this loss of function in early tumorigenesis, via early expansion of stem cells, would be predicted from recent data in *p16* knockout mice revealing that germ-line loss of this gene can increase stem cell life span (52–54). The rate of point mutations in *p16* in most cancer types is low, but the gene is a frequent target for early methylation in these same tumors, such as breast cancer and non-small cell lung cancer (55,56). This methylation occurs early in

tumor progression, prior to invasive cancer (55,57). In fact, histologically normal mammary epithelium from some healthy women without malignancy can harbor focal *p16* promoter hypermethylation (58). Experimentally, early loss of *p16* in mammary epithelial cells precedes genomic and epigenetic instability (59–61).

Another excellent example of the potential role for early epigenetic abnormalities and stem/precursor cell expansion to contribute to early steps in tumor progression involves colon cancer. In this disease, cancer risk can begin with appearance of aberrant crypt foci in the colonic epithelium and these harbor premalignant, hyperplastic, preadenomatous cells (62,63). The evolution of colon cancer is highly dependent on abnormal activation of the stem/precursor cell Wnt pathway, which by the time frank polyps and/or invasive lesions appear, is driven by classic inactivating mutations in the *APC* gene or activating mutations of β -catenin, key downstream players in the pathway (64,65). In aberrant crypt foci, however, such mutations may not be present, yet there is DNA hypermethylation (32,66) of a family of genes, the SFRPs, which encode for membrane region proteins that antagonize Wnt interaction with its receptors (66,67). This hypermethylation persists throughout colon tumor progression and can later collaborate with the downstream mutations in driving the Wnt pathway (32,66).

Translational Implications of Epigenetic Changes in Cancer

The delineation of epigenetic abnormalities in tumorigenesis is contributing not only to our understanding of the biology of cancer but is already having distinct implications for our management of these diseases. The overall abnormalities in chromatin

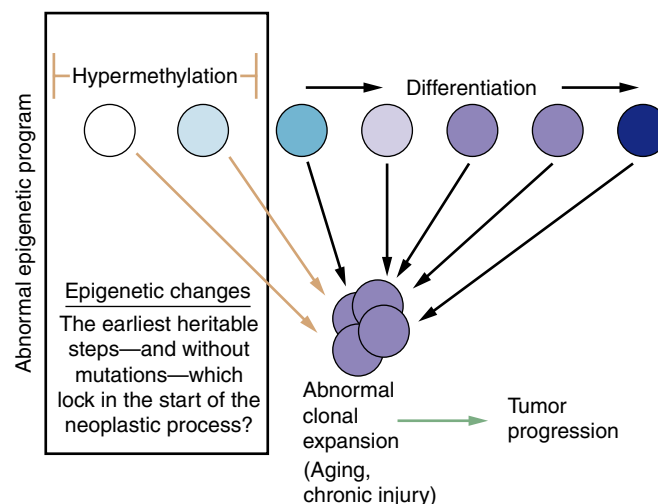


FIGURE 5-4 The potential that epigenetic gene-silencing events have for participation in the earliest stages of tumor progression. As discussed in the text and Figure 5-3, suppression of gene transcription can be a normal event for a group of key genes in stem cells and progenitor cells as adult epithelial-cell renewal takes place (left large box). This low-level gene expression is accomplished by a balance of chromatin modifications that associate with active and repressed transcription (bivalent chromatin; Figure 5-3), but transcription can increase in maturing cells during normal cell renewal. This balance of control for gene expression allows stem and progenitor cells to progress along a normal differentiation pathway (moving with arrow from left to right across the top of the figure). During chronic and abnormal pressures upon stem-cell and progenitor-cell pools for tissue repair, there is a tendency for the gene chromatin constituents in these cells (Figure 5-3) to recruit promoter DNA hypermethylation (top of large box) and this becomes associated with heritable silencing of the genes (abnormal epigenetic program, large box). This inability of the genes to increase with maturation cues facilitates abnormal clonal expansion of stem/progenitor cells (heavier arrows), at the expense of differentiation. Such expansion may occur in stroma, leading to an abnormal environment that helps support epithelial tumor growth. This process renders the abnormal clones at risk for further tumor progression (bottom arrow) driven by subsequent genetic or epigenetic events.

organization under discussion are beginning to provide potential biomarkers for use in cancer risk assessment, early diagnosis, and prognosis assessment. For example, during early stages of tumor progression, some of the histone modifications change in cancer cells (Figures 5-2 and 5-3) are manifest, as previously discussed, as changes in global levels of these parameters, such as losses of monoacetylated and trimethylated forms of histone H4, and losses of acetylated Lys16 and trimethylated Lys20 residues of histone H4 (45,68). Changes in modification marks on histones H3 and H4 have been correlated with aggressiveness of prostate cancer (68). These global changes are hypothesized to be common hallmarks of human tumor cells and might be developed as important biomarkers. Similarly, increases in levels of enzymes that catalyze key facets of cancer epigenetic abnormalities, such as the DNA methyltransferases for DNA methylation (41), and more recently histone methyltransferases such as EZH2 (43,44) for H3K27 methylation, and other PcG gene silencing constituents (69), have been correlated with several cancer types and correlated with aggressive behavior. The most developed biomarker strategies have been centered on the gene promoter DNA hypermethylation and gene silencing that has been a major focus of this chapter, and this will be discussed in more detail in the following sections.

Another major translational implication of cancer epigenetic abnormalities is for the prevention and therapy of these diseases. Particularly promising is the concept of targeting reversal of DNA hypermethylation and aberrant gene silencing as a strategy for these purposes (70). This goal is also discussed.

Developing Hypermethylation of Gene Promoter CpG Islands as a Molecular Marker Strategy for Cancer

Use of promoter–hypermethylation sequences as a molecular signature is providing one of the most promising biomarker strategies for cancer (71–73). One advantage of the approaches being adapted relies on the relative stability of DNA as compared with many proteins and RNA, which allows for use of paraffin-embedded clinical samples for detection strategies. Given the fact that, as discussed, the numbers of genes DNA hypermethylated is so high in individual tumors, and that this phenomenon is common in all cancer types, it is not difficult to build profiles of relatively small hypermethylated gene panels in which one or more markers are positive in virtually any cancer (74). Combined with a growing repertoire of sensitive polymerase chain reaction (PCR)–based assay procedures to specifically detect the hypermethylated sequences (72,73,75), and the fact that these assays can be targeted to constant positions of the abnormal CpG methylation in gene promoter regions, relatively simple detection strategies are being constructed. With such assays, abnormally methylated gene sequences have been detected in sources as diverse as DNA extracted from tumor, lymph nodes, serum, sputum, bronchial-lavage fluid, and urine for patients with all varieties of cancer types (71–73). The strategies range from determining whether the methylation patterns in tumors reflect prognosis for behavior, to use of marker detection in distal sites

for purposes of cancer risk assessment, early diagnosis, and staging. For example, studies of sputum DNA from patients at high risk for lung cancer have found that invasive tumors may be predicted, with odds ratios of 6 or more, more than a year before clinical detection of cancer (76) and findings of abnormal methylation markers in sputum may be useful for predicting which patients with surgically resected early stage lung cancers may recur (77). The occurrence of hypermethylation of specific genes in tumor DNA may predict future behavior of a cancer and reportedly, this change for the *p16* gene in DNA from lung cancers predicts high likelihood of poor outcome (78).

The promise of these approaches will be realized only from continued studies of ever-increasing size. The precise assays best suited for routine clinical use must be determined and approaches that build quantitative determinations into these assays (79) must be evaluated. Confounding issues must be continuously considered and a most critical one is to always consider whether the presence of hypermethylated gene markers in normal-appearing tissue settings means cancer risk versus actual cancer presence. This accentuates the importance of the information discussed earlier in this chapter concerning the biology of cancer as it involves epigenetic abnormalities. The position for appearance of individual gene markers in tumor progression is critical and must be paired with consideration of risk factors. For example, gene promoter methylation in normal tissues can increase with age, as best studied in the colon (80) and parallels the risk of cancer at a given site. All of this information defines the potential power of marker strategies using gene promoter hypermethylated sequences and the caveats that must be considered in using these strategies.

Perhaps one of the most promising uses for gene hypermethylation markers, and one, perhaps, closest to actual clinical realization, concerns their use for prediction of drug sensitivity. This strategy exploits the fact that aberrantly silenced genes involved with this epigenetic abnormality can belong to pathways that dictate cellular pathways integral to drug responsiveness. The most developed example of this is the silencing of the DNA repair gene, *O⁶-MGMT*, which encodes for a protein that mediates removal of bulky alkylation adducts from guanines (71). Several tumor types lose the function of *O⁶-MGMT* via aberrant silencing of the gene and constituent cells have a diminished capacity to repair alkylation damage, rendering them sensitive to alkylating agents such as temozolomide (81,82). Thus, multiple studies reveal that patients with brain tumors harboring *O⁶-MGMT* respond remarkably better to this agent than those whose tumors lack this change providing an exceptionally promising marker to stratify patients with this lethal tumor type for best therapy approaches (81,82). If ongoing trials continue to validate this, a relatively easy and rapid marker could reach actual clinical use.

Targeting Epigenetic Abnormalities for Cancer Prevention and Therapy

There is now growing appreciation that our expanding knowledge of epigenetic abnormalities in cancer, in general, and most especially, at present, the definition of aberrant gene silencing as an

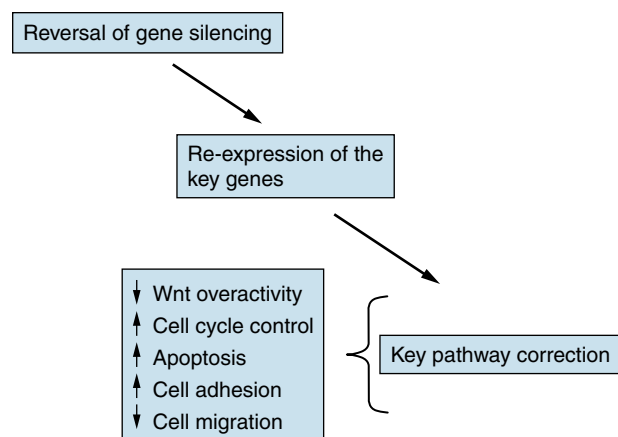


FIGURE 5-5 THE THEORY BEHIND EMPHASIZING TARGETING REVERSAL OF ABERRANT GENE SILENCING AS A STRATEGY FOR CANCER PREVENTION AND THERAPY. The concept is depicted that, based on the numbers of epigenetically silenced genes in a given tumor, the number of pathways affected by the epigenetically mediated loss of gene function and the network affects of the silencing within and between the pathways, the strategy of reactivating silenced genes presents a unique opportunity to counter, via a single therapy, virtually all the steps that drive tumorigenesis.

alternative mechanism to mutations for loss of tumor suppressor gene function, offers extraordinary potential for exploitation in managing cancer (70,71). First, there is the critical difference that, as compared with mutations, epigenetic gene silencing, as we have discussed in this chapter, is potentially reversible. Second, the growing list of molecular steps being defined as components of the silencing offers more individual and combinatorial targets for considering interventions. Third, the early position of aberrant gene in tumor progression makes reversal of the silencing an attractive target for prevention approaches. Also, the potential of the silenced genes to participate in tumor recurrence suggests that the adjuvant treatment arena may be an attractive area for epigenetic therapy. Fourth, and perhaps most important, the biology discussed in this chapter, including the high frequency of the gene silencing abnormality in all cancer types, the numbers of genes involved in individual tumors, and the critical pathways for cancer development in which the involved genes participate, makes reversal of gene silencing not only a rational target for therapy, but an essential one to consider (Figure 5-5). If successful, reversal of the entire gene silencing in a given patient's tumor could, with one targeted therapy approach, reverse virtually every key signal pathway involved in the initiation, progression, and maintenance of the cancer (Table 5-1 and Figure 5-5).

Where do we stand in this important cancer prevention/therapy endeavor? Indeed, drugs that reverse DNA demethylation, such as 5-azacytidine and 5-aza-2'-deoxycytidine and histone deacetylase inhibitors that target the histone deacetylation component of gene silencing are already in the clinic (70,71,83,84) and approved by the U.S. Food and Drug Administration (FDA) for certain diseases. The concept that initial use of aza-cytidines followed by administration of HDACi's may be synergistic for inducing re-expression of aberrantly silenced cancer genes is receiving attention and

encouraging early clinical results (85,86). It must be stressed, however, that it remains to be established to what degree the individual or combined effects of these drugs on their targets, DNMTs and HDACs, plays a role in their therapeutic efficacy in patients with the premalignant disease, myelodysplasia (MDS), related leukemias, and cutaneous lymphomas for the HDACi's (70,71,85,87–90). Encouraging results indicate that, at least some of the clinical effects are due to true reversal of epigenetic targets. First, clinical efficacy is being accomplished, especially for the aza-cytidines, at far lower doses than the ones initially used. This results in much less of the toxic effects that may be due to nonepigenetic effects of the drugs, such as DNA damage (91,92). Second, emerging data suggest that the efficacy of the aza-cytidines correlates with the acute reversal of gene silencing. Early reactivation of the silenced cyclin-dependent kinase inhibitor encoding gene, *p15*, in myelodysplastic and leukemias, appears to correlate with subsequent patient responses in one study (85), although others have not found such correlation even though the gene is clearly re-expressed in patients' tumor cells during acute drug treatment (86).

Despite these encouraging developments, much remains to be done if epigenetic therapies can make a powerful impact on the prevention and treatment of cancer. First, little efficacy for the common solid tumors has been shown. However, most attempts to treat these tumors occurred before it was appreciated that lower, and less toxic, doses of drugs such as the aza-cytidines and HDACi's can be used. The time is ripe for the regimens showing such promise in the liquid tumors to be applied to treatment of solid tumors. Second, despite the lessening toxicities now being seen for drugs such as the aza-cytidines, new classes of inhibitors of the DNMTs may be needed that do not incorporate into DNA and, for ease of patient use, can be administered orally. Third, as discussed previously in this chapter, our increasing knowledge of the chromatin components of gene DNA hypermethylation associated gene silencing, must be exploited. As shown in Figure 5-3, the retention of key silencing chromatin marks for re-expressed genes following promoter DNA demethylation predicts that, as experimentally seen (46,93), once administration of drugs such as the aza-cytidines are stopped, the silencing will return. Thus, feasibility for prolonged drug regimens may need to be shown and, indeed, such chronic administration appears possible for the aza-cytidines (89,90). Finally, the other chromatin components of the aberrant gene silencing represent additional drug targets that may enrich therapy possibilities. For example, unlike for the dominant role of the DNA methylation in the silencing over the participation of the class I and II HDACs, the interaction of the class III HDAC, SIRT1, may lie downstream of the methylation (94). Thus, inhibition of the activity of this protein appears to cause reactivation of aberrantly silenced cancer genes without necessity for removal of the promoter DNA hypermethylation (94). Many other of the chromatin steps previously discussed might be similarly exploited. The era is an exciting one for realizing major impact on cancer control resulting from the current explosion of knowledge about chromatin and gene regulation and of the role of epigenetic abnormalities in the initiation and progression of cancer.

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Infectious Agents and Cancer

Overview of Cancer and Infectious Agents

Infectious agents are second only to tobacco use as a potentially preventable cause of cancer in humans. Estimates vary between 15% and 30% as to the percentage of cancers worldwide that are associated with an infectious etiology (1,2). The burden is greater in the developing world but impact even in the United States and other developing countries is significant. Specific viruses, parasites, and bacteria are associated with specific human cancers, and these will be discussed in some detail in this chapter.

There are three major mechanisms by which an infectious agent can cause a cancer and these may involve the initiation and the promotion of carcinogenesis (3). The first is perhaps the most common and results from the infectious agent causing a persistent infection with chronic inflammation. This can result in the formation of reactive oxygen and nitrogen species by macrophages at the site of the infection. These reactive molecules can damage DNA, proteins, and membranes, and as such contribute to carcinogenesis (4). Chronic inflammation due to the persistent infection can lead to repeated cycles of cell damage and cellular proliferation. Cells that are cycling in the presence of reactive molecules are more likely to accumulate genetic mutations that could contribute to the initiation and promotion of cancer. A second mechanism involves the direct participation of the infectious agent in the transformation of the cell through the activation of a cellular oncogene pathway or the inactivation of a tumor suppressor gene. A third mechanism that is relevant to the human immunodeficiency virus (HIV) is that the infection may result in immunosuppression and the decreased recognition of the infected or transformed cell by the host immune system. Indeed many of the cancers observed in immunosuppressed patients, such as those infected with HIV, often are those that have been associated with other viruses.

The recognition of an infectious cause for specific cancers provides the opportunities to prevent the cancers by preventing or controlling the infections. Depending on the infectious agent, this could involve public health measures or changes in cultural practices. It could also involve the development of vaccines to prevent the initial infections, as has been achieved for hepatitis B virus (HBV) or the genital tract human papillomaviruses (HPVs). It could also involve the treatment of the infections with specific

therapeutics or the development of novel therapies for those agents for which there are not yet specific or effective drugs.

Viruses and Cancer

History of Viral Oncology

Viral oncology has its beginnings as a scientific discipline from observations made during the early part of this century when the transmissibility of avian leukemia was first described by Ellermann in Denmark in 1908 and the transmissibility of an avian sarcoma in chickens was described by Rous in 1911 (5,6). The importance of these findings was not appreciated at the time, and the full impact on virology and medicine was not recognized until the 1950s. Indeed the work of Peyton Rous (6) showing that cell-free extracts containing a filterable agent from a sarcoma in chickens could induce tumors in injected chickens within a few weeks was finally recognized by a Nobel prize in 1968. Rous' original work not only pointed out that a filterable agent (the working definition of a virus at that time) was capable of inducing tumors, but was also responsible for determining the phenotypic characteristics of the tumor. Since these studies were carried out in birds and not in mammals, however, this early work was consigned to the rank of avian curiosities.

In the 1930s, Richard Shope published a series of papers demonstrating cell-free transmission of tumors in rabbits. The first studies involved fibromatous tumors found in the footpads of wild cottontail rabbits that could be transmitted by injecting cell-free extracts in wild or domestic rabbits—a virus referred to as the Shope fibroma virus is now known to be a pox virus. Other studies carried out by Shope demonstrated that cutaneous papillomatosis in wild cottontail rabbits could also be transmitted by cell-free extracts. In a number of cases, these benign papillomas would progress spontaneously into squamous cell carcinomas in infected domestic rabbits or in the infected cottontail rabbits (7,8). In general, however, the field of viral oncology lay dormant until the early 1950s, with the discovery of the murine leukemia viruses by Ludwig Gross (9) and of the mouse polyoma virus by Gross, Stewart, and Eddy (10,11). These findings of tumor viruses in mice led many cancer researchers and virologists to the field of viral oncology. These researchers had the hope that these

initial observations in mammals could be extended to humans and that a fair proportion of human tumors might also be found to have a viral etiology. The Special Viral Cancer Program at the National Cancer Institute grew from this intense interest in viral oncology and the hope that human tumor viruses would be identified.

Many of the most important developments in modern molecular biology derive from studies in viral oncology from the 1960s and 1970s. The discovery of reverse transcriptase, the development of recombinant DNA technology, the discovery of messenger RNA splicing, and the discovery of oncogenes and, more recently, tumor suppressor genes all have been developments that derive directly from studies in viral oncology. Oncogenes were first recognized as cellular genes that had been acquired by retroviruses through recombinational processes to convert them into acute transforming RNA tumor viruses. It is now recognized that oncogenes participate in many different types of tumors and can be involved at different stages of tumorigenesis and viral oncology. This has contributed significantly to our concepts in nonviral carcinogenesis. It is likely that the direct transforming, oncogene-transducing retroviruses do not play a major causative role in naturally occurring cancers in animals or in humans, but rather represent laboratory-generated recombinants. A list

of human viruses with oncogenic properties is presented in Table 6-1. This list includes viruses such as the transforming adenoviruses, which are capable of transforming normal cells into malignant cells in the laboratory but have not been associated with any known human tumors. The list also includes viruses such as the human T-cell leukemia virus type 1 and some of the human papillomaviruses that have been etiologically associated with specific human cancers and shown to encode transforming viral oncogenes. It also includes human viruses such as HBV and hepatitis C virus (HCV), which are clearly associated with hepatocellular cancers in humans, but may not encode transforming genes, and which may contribute to carcinogenesis through persistent infection and causing chronic inflammation.

Also listed in Table 6-1 are cofactors that are believed to be important in the carcinogenic processes associated with each of these viruses. It is clear that none of these viruses by themselves is sufficient for the induction of the specific neoplasias with which they have been associated. Each of the viruses associated with these human cancers is thought to be involved at an early step in carcinogenesis. Subsequent cellular genetic events such as somatic mutations are thought to then be important at the subsequent steps involved in multistep process of malignant progression.

Table 6-1 Human Viruses with Oncogenic Properties

Virus Family	Type	Human Tumor	Cofactors	Comments
Adenovirus	Types 2, 5, 12	None	N/A	Important experimental model
Hepadnavirus	Hepatitis B (HBV)	Hepatocellular carcinoma (HCC)	Aflatoxin, alcohol, smoking	Causative
Herpesvirus	Epstein-Barr (EBV)	Burkitt lymphoma	Malaria	EBV considered a cofactor
		Lymphoproliferative disease	Immunodeficiency	Causative
		Nasopharyngeal carcinoma	Nitrosamines, genetic	Causative
		Hodgkin lymphoma	Unknown	Variable association
	KSHV (HSV-8)	Kaposi sarcoma	AIDS	Causative
		Castleman disease	Unknown	Causative
		Primary effusion lymphomas	Unknown	Causative
Flavivirus	Hepatitis C virus	Hepatocellular carcinoma	Aflatoxin	Causative
Papillomaviruses	HPV-16, -18, -31, -33, -35, -39 and others	Anogenital cancers and some upper airway cancers	Smoking, oral contraceptives, ? other factors	Causative
	HPV-5, -8, -17, -20, -47	Skin cancer	Genetic disorder (EV), UV	Unclear if causative
Polyomavirus	BK	?Prostate preneoplastic lesions	N/A	Unclear if causative
	JC	?Brain tumors	N/A	Unclear if causative
	SV40 ^a	?Mesotheliomas, brain tumors, etc.	N/A	Unlikely
Retroviruses	HTLV-1	ATL	?Genetic	Causative
	HTLV-2	None	N/A	Not associated with human malignancy

ATL, adult T-cell lymphoma; N/A, not applicable; SV40, simian virus 40.

^aSV40 is a nonhuman primate virus closely related to the human polyomaviruses BK and JC.

Table 6-2 Association of HPVs and Clinical Lesions

A. CUTANEOUS LESIONS AND HPVs CLINICAL ASSOCIATION VIRAL TYPES	
Plantar wart	HPV-1
Common wart	HPV-2, -4
Mosaic wart	HPV-2
Multiple flat warts	HPV-3, -10, -28, -41
Macular plaques in EV	HPV-5, -8, -9, -12, -14, -15, -17, -19, -20, -21, -22, -23, -24, -25, -36, -47, -50
Butcher's warts	HPV-7
B. GENITAL TRACT AND OTHER MUCOSAL HPVs	
Condyloma acuminata (exophytic)	HPV-6, -11
Giant condyloma (Bushke-Lowenstein tumor)	HPV-6, -11
Subclinical infection	All genital tract HPV types
Squamous intraepithelial lesions	HPV-16, -18, -31, -33, etc.
Bowenoid papulosis	HPV-16
Cervical cancer	
Strong association "high risk"	HPV-16, -18, -31, -45
Moderate association	HPV-33, -35, -39, -51, -52, -56, -58, -59, -68
Weak or no association "low risk"	HPV-6, -11, -26, -42, -43, -44, -51, -53, -54, -55, -66
Other anogenital cancers (vulvar, penile, etc.)	HPV-16
Respiratory papillomas	HPV-6, -11
Conjunctival papillomas	HPV-6, -11
Focal epithelial hyperplasia (oral cavity)	HPV-13, -32
Upper airway and tonsillar cancer	HPV-16

EV, epidermodysplasia verruciformis; HPV, human papillomavirus.

DNA replication for progeny virion production can be detected by in situ hybridization only in the more differentiated squamous epithelial cells of the stratum spinosum and of the granular layer of the epidermis, but not in the basal layer nor in the underlying dermal fibroblasts. Viral capsid protein synthesis and virion assembly occur only in the terminally differentiated cells of the upper layers of the epithelium. The viral genome is present in the epithelial cells of all layers of the epithelium, including the basal layer. It is generally believed that the expression of specific viral genes in the basal layer and in the lower layers of the epidermis stimulates cellular proliferation and alters the keratinocyte differentiation profile, characteristics of a wart. As squamous epithelial cells migrate upward through the layers, they undergo a program of differentiation. The control of papillomavirus late-gene expression is tightly linked to the differentiation state of the squamous epithelial cells. The basis of this control is not yet fully understood, but involves the activation of a "late" promoter and altered patterns of viral messenger RNA processing (13).

The genomic organization of all the PVs is quite similar. All of the open reading frames (ORFs) that could serve to encode proteins for these viruses are located on only one of the two viral DNA strands and only one strand is transcribed. The HPV genome can

be divided into three distinct regions: (1) an "early" region that encodes the viral proteins (E1, E2, etc.) involved in viral DNA replication, transcriptional regulation, and cellular transformation; (2) a "late" region that encodes the viral major (L1) and minor (L2) capsid proteins; and (3) a region called the long control region (LCR) or alternatively, the upstream regulatory region (URR), which does not contain any ORFs, but does contain *cis*-regulatory elements, including the origin of DNA replication and important transcriptional enhancers. A diagram of the organization of the HPV-16 genome, which is typical of all the HPVs, is shown in Figure 6-2. In productive infections (i.e., infections in which viral particles), messenger RNA is transcribed from the early and late regions of the genome. However, the late genes (L1 and L2) are only expressed in the more differentiated cells of the epithelium. The nonproductive phase of the infectious cycle occurs in the less differentiated cells in the lower epithelium and is accompanied by expression of the early (E) region genes.

Papillomaviruses and Cancer

Only a subgroup of the PVs is associated with cancer. These include several animal PVs and some HPVs (listed with their associated cancers in Table 6-3) in addition to the cotton-tail rabbit papillomavirus (CRPV) first identified by Richard Shope as the etiologic agent of cutaneous papillomatosis in rabbits (16). CRPV has been extensively studied as a model for PV-induced carcinogenesis. One of the principle features of the carcinogenic progression associated with the PVs, is the synergy observed between the virus and carcinogenic external factors (Table 6-3). In the case of CRPV, carcinomas develop at an increased frequency in papillomas that are painted with cool tar or with methylcholanthrene (17,18). These

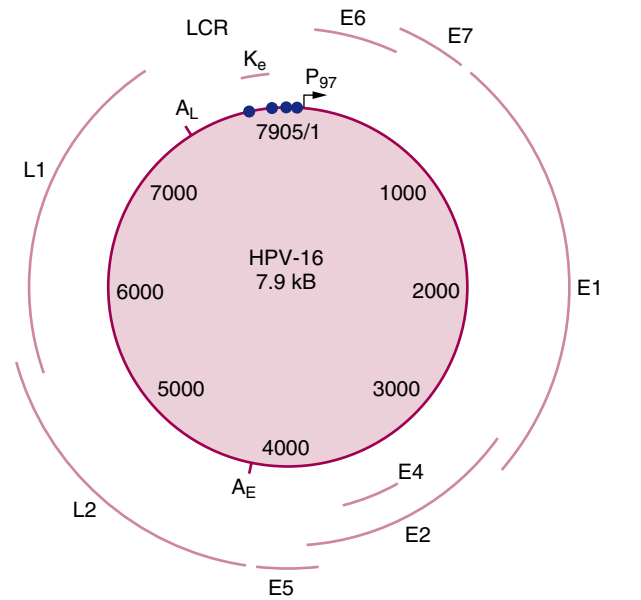


FIGURE 6-2 MAP OF THE HPV-16 GENOME. The nucleotide numbers are noted within the circular maps, transcription proceeds clockwise, and the major open reading frames (E1 to E7, L1, and L2) are indicated. The transcriptional promoter that directs the expression of E6 and E7 is designated (P_{97}). A_E and A_L represent the polyadenylation signals for the early and late transcripts, respectively. The viral long control region (LCR) contains the viral transcriptional and replication regulatory elements. The closed circles on the genome represent the four E2 binding sites that have been noted in the LCR. (Modified from Ref 14, with permission.)

Table 6-3 Papillomaviruses Associated with Cancers in Natural Host

Species	Cancers	Viruses	Other Factors
Human	Anogenital cancers	HPV-16, -18, -31, etc.	Smoking
	Oral and tonsillar cancers	HPV-16	
	Malignant progression of respiratory papillomas	HPV-6, -11	X-irradiation, smoking
	Skin cancer	HPV-5, -8, -17, etc.	Genetic (EV), UV light, and immunosuppression
Rabbit	Skin cancer	CRPV	Methylcholanthrene and coal tar (experimental)
Cattle	Alimentary tract cancers	BPV-4	Bracken fern
	Ocular cancers	Not characterized	UV light
Sheep	Skin cancer	Not characterized	UV light

EV, epidermodysplasia verruciformis; UV, ultraviolet.

CRPV-associated carcinomas contain the viral DNA that is transcriptionally active, and the carcinogenic properties are believed to map to specific viral genes.

In cattle, BPV4 causes esophageal papillomatosis and is associated with squamous cell carcinomas of upper alimentary tract (19). Interestingly, only those cattle from the highlands of Scotland that feed on bracken fern (known to contain a radiomimetic substance) and that are also infected by BPV4, have a high incidence of squamous cell carcinomas of the esophagus and of the foregut (19). In contrast to the CRPV-associated carcinomas in which the viral DNA can invariably be found, extensive analysis of the squamous cell carcinomas of the upper alimentary tract in these cattle infected with BPV4 have failed to reveal a consistent pattern of viral DNA sequences within the malignant tumors (19, 20). In the case of these alimentary tract tumors, it is possible that the continued presence of BPV4 DNA sequences is not required for the maintenance of the cancer.

HPVs have been linked to cervical cancer and other anogenital cancers as well as to some upper airway oropharyngeal cancers and nonmelanoma skin cancers. These will be discussed in the following sections.

HPV and Cervical Cancer

Cervical cancer is the second most common malignancy among women worldwide, with approximately 500,000 newly diagnosed cases each year and about 275,000 deaths annually (1,2). About 80% of cervical cancer occurs in developing countries, where it is frequently the most common cancer of women, accounting for as many as one fourth of female cancers. It occurs less frequently in developed countries. In the United States, there are about 12,000 newly diagnosed cases annually, and about one third of these women will die of their malignant disease. The incidence of cervical cancer in the United States varies considerably between ethnic and socioeconomic groups, with the rate among black women about twice that of white women (21).

Most cancers occur in the transformation zone of the cervix, where the columnar cells of the endocervix form a junction with the stratified squamous epithelium of the exocervix. About 85% of

cervical cancers are squamous cell cancers, the remainder are adenocarcinomas and small cell neuroendocrine tumors. The progression of normal cervical epithelial cells to malignant squamous cell carcinomas typically progresses through a series of dysplastic changes over many years, a process that is the basis of the Pap smear screening program. The histologic classification of cervical intraepithelial neoplasia (CIN), grades 1, 2, and 3 correspond, respectively, to mild dysplasia, moderate dysplasia, and severe dysplasia or carcinoma in situ. Because of the long interval for the progression of cervical dysplasia to invasive cancer, Pap smear screening programs can identify most premalignant lesions for appropriate treatment, thereby preventing the development of most cases of cervical cancer in countries with screening programs.

For decades, it has been recognized that cervical cancer is linked to a sexually transmitted agent, long before sexually transmitted HPV infection was implicated in its pathogenesis (22,23). Venereal transmission of a carcinogenic factor with a long latency had been suggested by the early epidemiologic studies. Sexual promiscuity, an early age of onset of sexual activity, and poor sexual hygienic conditions were identified as risk factors in women for cervical carcinoma. The counterpart to cervical cancer in the male appears to be penile cancer because there is a correlation between the incidence rates of these two cancers in different geographic areas. The incidence rates for penile carcinoma; however, are on the order of 20-fold lower than those of cervical carcinoma. However, the similar ratio of incidence between cervical and penile carcinoma is maintained in areas of high, medium, or low prevalence, suggesting that the etiologic factors for penile and cervical carcinoma may be the same.

Compelling evidence linking an HPV infection with cervical carcinoma followed the observation that some of the morphologic changes characteristic of cervical dysplasia observed on Pap smears were due to a papillomavirus infection (24–26). The cell with its characteristic perinuclear clearing and abnormally shaped nucleus that is diagnostic for a cervical PV infection is the koilocyte. The presence of PV particles, PV-specific capsid antigens, and HPV DNA within the cervical preneoplastic lesions provided confirmation of the HPV etiology of cervical dysplasia.

The association of an HPV with the preneoplastic lesions of the cervix prompted the search for HPV sequences in cervical

cancers. zur Hausen and colleagues identified the first papilloma-virus DNAs, HPV-16 and HPV-18, in cervical cancer tissues (27,28). Using the HPV-16- and HPV-18-cloned DNAs as probes, approximately 70% of cervical carcinomas were shown to harbor these two HPV DNAs (29). Subsequent studies led to the identification of approximately 30 different HPVs associated with genital tract lesions, a subset of which are associated with human cervical cancer. In addition to HPV-16 and HPV-18, several of the other genital tract-associated HPVs (HPV-31, HPV-33, HPV-39, HPV-45, among others) have been associated with cervical carcinomas. Specific HPVs are found in approximately 90% of human cervical carcinomas (30). DNAs from these same HPV types are found in other human genital carcinomas including penile carcinomas, some vulvar carcinomas, and some perianal carcinomas, and in the precancerous intraepithelial lesions of each of these sites (penile intraepithelial neoplasia [PIN], vulvar intraepithelial neoplasia [VIN], and perianal intraepithelial neoplasia [PAIN]). The availability of HPV DNA probes has permitted the extensive analysis of specific clinical lesions.

The genital tract-associated HPVs have been classified as “high risk” or “low risk” on the basis of whether the lesions with which they are associated are at significant risk for malignant progression. The low-risk viruses such as HPV-6 and HPV-11 are associated with venereal warts, lesions that only rarely progress to cancer. The high-risk viruses such as HPV-16 and HPV-18 are associated with CIN and cervical cancer. The other high-risk viruses include HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-68, and HPV-82. As noted previously, approximately 90% of cervical carcinomas contain a high-risk DNA. HPV-positive cervical cancers and cell lines derived from HPV-positive cervical cancer tissues often contain integrated viral DNA, although there are some cases in which DNA is apparently also extrachromosomal. In those cancers in which the viral DNA is integrated, the pattern of integration is clonal, indicating that the integration event preceded the clonal outgrowth of the tumor. Integration of the viral DNA does not occur at specific sites in the host chromosome, although in some cancers the HPV DNA has integrated in the vicinity of known oncogenes. For instance, in the HeLa cell line (which is an HPV-18-positive cervical carcinoma cell line), integration of the HPV-18 genome is within approximately 50 kb of the *c-myc* locus on human chromosome 8. It is possible that such an integration event might provide a selective growth advantage to the cell, and as such, might contribute to neoplastic progression.

The Role of HPV in Cervical Cancer

In cervical cancers, only a subset of the viral genes is expressed, and there is no production of virus by the cancer cells. The integration of the viral genome appears to play an important role leading to the deregulated expression of the viral E6 and E7 genes (13). The E6 and E7 genes are invariably expressed in HPV-positive cervical cancers. Integration of the HPV genome into the host chromosome in the cancers often results in the disruption of the viral E1 or E2 genes. Since HPV E2 is a viral regulatory factor that negatively regulates expression of the E6 and E7 genes, the disruption

of E2 results in the derepression of the E6/E7 promoter, leading to deregulated expression of E6 and E7. Indeed, the introduction of E2 into cervical cancer cell lines results in the induction of cellular senescence by repressing E6 and E7 expression.

The E6 and E7 genes of the high-risk genital-tract associated HPVs are transforming genes. E7 is by itself sufficient for the transformation of established rodent cells such as NIH 3T3 cells and can cooperate with an activated *ras* oncogene to transform primary rodent cells (31). Expression of E6 and E7 together are sufficient for the efficient immortalization of primary human cells, most notably, primary human keratinocytes, which are the normal host cell for the HPVs (32,33). In contrast to the immortalization properties of the HPV-16 and HPV-18 E6 and E7 proteins, the E6 and E7 proteins encoded by the low-risk viruses are inactive or only weakly active in the same assays. A number of studies have suggested that the HPV E5 genes may also have transforming activities in a variety of assays; however, E5 is usually not expressed in the cancer cells. Therefore, if E5 has a role in cervical carcinogenesis, it must be at an early stage because its expression is not necessary to maintain the cancer.

The major cellular targets for E6 and E7 are the tumor suppressor proteins p53 and pRB, respectively. E6 and E7 are polyfunctional proteins and have many other biochemical activities and biologic properties that may be relevant to their activities in cervical carcinogenesis (13). A common theme among the small DNA tumor viruses (i.e., the polyomaviruses, the adenoviruses, and the cancer-associated HPVs) is that the immortalization and transformation properties of their encoded oncoproteins are in part due to their interactions with critical cellular regulatory proteins (Figure 6-3). The E7 proteins encoded by the high-risk HPVs share some amino-acid sequence similarity to adenovirus E1A and portions of the SV40 large, T-antigen in regions that are critical for the transformation activities of these oncoproteins. These regions of amino acid sequence similarity shared by these viral oncoproteins are regions that specify the binding to a number of important cellular regulatory proteins, including the product of the retinoblastoma tumor suppressor gene, pRB, and the related

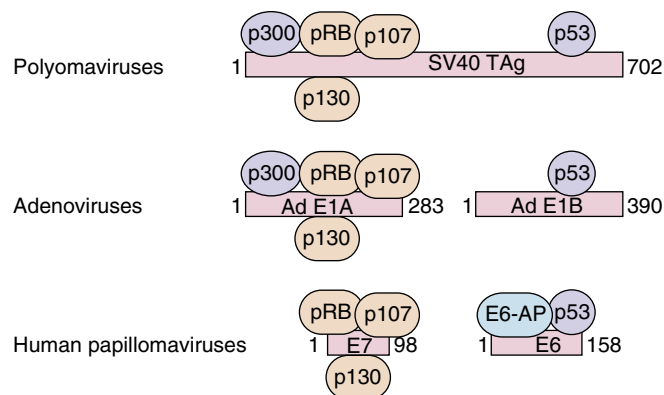


FIGURE 6-3 THE TRANSFORMING PROTEINS ENCODED BY THREE DISTINCT GROUPS OF DNA TUMOR VIRUSES TARGET SIMILAR CELLULAR PROTEINS. The binding of human papilloma-virus (HPV) E6 oncoproteins to p53 is mediated by the cellular protein called E6AP, which functions as an E3 ubiquitin protein ligase to target the E6-dependent ubiquitylation of p53. (Modified with permission from Ref 15.)

pocket proteins p107 and p130 (34–36). Studies have established that a major component of the transformation activities of these viral oncoproteins is due to their respective abilities to complex and functionally inactivate pRB and the related “pocket proteins” (37). The binding of these viral oncoproteins to pRB, p107, and p130 leads to cellular proliferation through the activation of genes under the control of the E2F family of transcription factors. The transcriptional activities of the individual members of the E2F family of transcription factors are modulated by pRB and the other pocket proteins. When complexed with E2F proteins, they act as transcriptional repressors, and when dissociated from the pocket proteins by E7, E1A, or SV40 T-antigen, E2F can act as a transcriptional activator. Consistent with this model, overexpression of E2F results in cell-cycle progression and can induce morphologic changes in cultured cells that are characteristic of cellular transformation. In the normal life cycle of the PVs, the binding of E7 to pRB is apparently essential for the activation of the cell-cycle DNA replication machinery in differentiated keratinocytes that had otherwise exited the cell cycle. The small DNA tumor viruses, including HPV, depend on the host-cell DNA polymerases and replication machinery for the replication of their viral genomes. Since this machinery is only expressed in the S phase of the cell cycle, these viruses must stimulate cellular proliferation and drive the cell into the S phase. In the case of HPV, it does so through the binding of E7 to pRB to free up the E2F family of transcription factors.

Genetic studies indicate that complex formation between E7 and the pocket proteins, including pRB, is not sufficient to account for its immortalization and transforming functions, suggesting that there are likely to be additional cellular targets of E7 that are relevant to cellular transformation (38). Indeed a large number of putative cellular targets for E7 have been identified (13) using a variety of biochemical approaches; however, the physiologic relevance of many of these interactions is not yet clear.

Some of these targets appear to be relevant to cervical cancer. For instance, E7 can interact with cyclin-dependent kinase inhibitors. Like Ad E1A, HPV-16 E7 interacts with and abrogates the inhibitory activity of p27^{kip1} (39). Since p27^{kip1} is involved in mediating the cellular growth inhibition by TGF- β in keratinocytes, this activity may contribute to the ability of E7 to override TGF- β -associated growth arrest (40). HPV-16 E7 can also associate with p21^{cip1} and abrogate its inhibition of cdks as well as its inhibition of PCNA-dependent DNA replication (41,42). p21^{cip1} is normally induced during keratinocyte differentiation (43), and inhibition by E7 may be critical in allowing the replication of papillomavirus DNA in differentiated squamous epithelial cells (44).

In addition, the high-risk HPV E7 proteins cause genomic instability in normal human cells (45). HPV-16 E7 induces G₁/S and mitotic cell-cycle checkpoint defects and uncouples synthesis of centrosomes from the cell-division cycle (46). This causes formation of abnormal multipolar mitoses, leading to chromosome mis-segregation and aneuploidy (47). Moreover, there is an increased incidence of double-strand DNA breaks and anaphase bridges, suggesting that in addition to numeric abnormalities, high-risk E7 proteins induce structural chromosome aberrations (48). Abnormal centrosome duplication rapidly results in genomic

instability and aneuploidy, one of the hallmarks of a cancer cell. This activity is therefore likely to be functionally relevant to the contribution of high-risk HPVs to malignant progression.

The immortalization/transformation properties of the E6 protein were first revealed by studies using primary human genital squamous epithelial cell (32,33). Efficient immortalization of primary human cells by HPV-16 or HPV-18 requires both the E6 and E7 genes. Like SV40 large T-antigen and the 55-kd protein encoded by adenovirus E1B, the E6 proteins of the high-risk HPVs can enter into a complex with p53 (49). The interaction of E6 with p53 is not direct but is mediated by a cellular protein, called the E6-associated protein (E6AP)(50). E6AP is a ubiquitin protein ligase and, in the presence of E6, directly participates in the ubiquitination of p53 (51). Multiubiquitinated p53 is then recognized and degraded by the 26S proteasome. Consequently the half-life and level of p53 are low in E6-immortalized cell lines and HPV-positive cancers. Through its ubiquitination of p53, HPV 16 E6 can abrogate the transcriptional activation and repression properties of p53 and disrupt the ability of wild-type (wt) p53 to mediate cell-cycle arrest in response to DNA damage. The p53 protein can sense DNA damage and prevent the replication of mutated DNA through its transcriptional activation of the p21 cyclin-dependent kinase inhibitor. Thus, the functional abrogation of p53 by high-risk HPV E6 results in decreased genomic stability and accumulation of DNA abnormalities in high-risk HPV E6-expressing cells. Hence, E6 can be directly implicated in the establishment and propagation of genomic instability, a hallmark in the pathology of malignant progression of cervical lesions.

The development of centrosome abnormalities and aneuploidy, two important related pathologic processes, appear to be initiated before viral DNA integration and may contribute to this process (52). High-risk HPV can induce abnormal centrosome duplication, which can result in genomic instability and aneuploidy (48). The deregulation of this mitotic event appears to depend on both E6 and E7, with the latter protein being most responsible for the effect. Indeed the deregulated viral oncogene expression may result in chromosomal instability and aneuploidy, enhancing the likelihood of viral DNA integration.

A number of additional cellular targets have been identified for the high-risk E6 proteins in an attempt to define additional p53-independent cellular targets. The reader is referred to *Fields Virology* (13) for a more comprehensive discussion of these additional activities, some of which may be relevant to the role of E6 in cervical carcinogenesis. Two activities are of particular importance however and will be discussed here. The first is the binding to cellular PDZ domain containing proteins. Interestingly the high-risk E6 oncoproteins contain an X-(S/T)-X-(V/I/L)-COOH motif at the extreme C-terminus that can mediate the binding to cellular PDZ domain containing proteins. This motif is unique in the high-risk HPV E6 proteins and is not present in the E6 proteins of the low-risk HPV types. E6 serves as a molecular bridge between these PDZ domain proteins and E6AP, facilitating their ubiquitylation and mediating their proteolysis. Among the PDZ domain proteins implicated as E6 targets are hDlg, the human homologue of the *Drosophila melanogaster* disc large tumor suppressor, and hScrib, the human homologue of the *Drosophila scribble* tumor suppressor (53,54).

Additional PDZ domain proteins have also been shown to be capable of binding to E6. Several PDZ-containing proteins have been shown to be involved in negatively regulating cellular proliferation. Therefore, some of the p53-independent transforming activities of the high-risk E6 oncoproteins may be linked to their ability to bind and degrade some of these PDZ motif-containing proteins.

The second important p53-independent activity of HPV-16 E6 is its ability to activate telomerase in keratinocytes through the transcriptional up-regulation of the rate-limiting catalytic subunit of human telomerase (hTERT) (55,56). Maintenance of telomere length is an important step in cancer and can occur through the transcriptional activation of hTERT expression or activation of the ALT recombination pathway. Activation of hTERT is observed in most human cancers, including HPV-positive cervical cancers. The mechanism by which E6 activates the hTERT promoter has not been yet fully elucidated but could involve the direct activation of a cellular transcription factor by E6 or the E6AP-dependent degradation of a negative regulator of the hTERT promoter.

It is clear that HPV infections by themselves are not sufficient for carcinogenic progression. Cancer is a rare outcome of infection, even with a high-risk HPV, such as HPV-16 or HPV-18. Expression of the E6 and E7 oncogenes is therefore not sufficient for malignant progression. Moreover, the time period between HPV infection and the development of invasive cancer can be several decades. Thus, infection with a high-risk HPV constitutes only one step in cervical carcinogenesis, and the genetic information carried by the virus per se is not sufficient to cause cancer. Epidemiologic studies have suggested that smoking is a risk factor for developing cervical carcinoma (57). The recognition that other factors are involved in the progression to cervical carcinomas suggests that papillomavirus infections may work synergistically with these other factors.

Specific chromosomal abnormalities have been detected in cervical cancer, including the loss of heterozygosity on the short arm of chromosome 3 (3p21) (58). This locus contains the FHIT gene (59), however, reintroduction of FHIT into tumor cells did not alter their biologic activity. Tumor progression is a complex process that involves multiple additional genetic loci. One possibility is that cellular mutations or epigenetic changes could be involved in down-regulating HLA antigen class I alleles and the ability of an HPV-positive cancer cell to be recognized by the host cellular immune response.

HPV and Other Cancers

Specific HPV DNA probes have been used by many investigators to carry out extensive screening of a many different human cancers for HPV sequences. Based on the animal models, any squamous carcinomas or cancers originating from an epithelium that has the potential to undergo squamous metaplasia is a candidate for an association with HPV.

HPV is linked to some head and neck cancers, although not to most cancers in this region. HPV-16 accounts for about 90% of the HPV-positive tumors. Most of these HPV-associated cancers are located in the oral pharynx, which includes the tonsils, tonsillar fossa, base of the tongue, and soft palate. In the United States, the incidence of these cancers, but not those at other oral

sites, has increased approximately 2% per year between 1973 and 1995, presumably because of the increase in sexually transmitted HPV infection (60). Genital-oral sex may be a risk factor for these tumors, and the risk of HPV infection and cigarette smoking may be more than additive. HPV-positive tumors tend to have characteristic basaloid pathology, are less likely to harbor mutations of p53 or pRb, and are more likely to express p16.

Esophageal carcinomas in humans have also been reported to have some association with HPVs; however, the data are not as convincing as they are with the anogenital cancers and with some of the oral and upper airway cancers. The esophagus is lined by a squamous epithelium, and squamous cell papillomas of the esophagus have been described in humans. Additional studies are warranted to investigate a possible role of HPV in human esophageal cancers. There have also been sporadic reports associating occasional human tumors, including colon cancer, ovarian cancer, prostate cancer, and melanomas, with the presence of HPV DNA in the literature. In general, it seems prudent to be skeptical of such reports until systematic and well-carried-out studies are confirmed in multiple laboratories.

Epidermodysplasia Verruciformis

Epidermodysplasia verruciformis (EV) is a rare disorder in which affected individuals have a unique susceptibility to cutaneous HPV infection (61,62). The warts usually develop in childhood, become widespread, do not tend to regress, and in approximately 30% of patients, progress to squamous cell cancers. Several types of lesions may occur in the same patient. Some lesions are typical flat warts (usually caused by HPV-3 or HPV-10) whereas others are flat, scaly, red-brown macules. The scaly lesions are associated with EV-specific HPV types, most frequently HPV-5 and HPV-8.

In approximately one half of affected patients, EV occurs as an inherited disorder. Inheritance appears to have an autosomal recessive pattern in most affected families, although one family with apparent X-linked recessive inheritance has been reported. Cases with autosomal recessive inheritance appear to be genetically heterogeneous, as the condition in different families has been mapped to two distinct chromosomal loci (63) and two adjacent novel genes (*EVER1* and *EVER2*) have now been molecularly identified at one of these loci (17q25) (64).

Patients with EV do not have an increased susceptibility to clinical infection with other microbial agents, including other HPVs. The EV-specific HPV types have now been found in normal skin of many individuals, so patients with EV are unusual in that these HPV types produce clinically apparent lesions. However, clinical lesions associated with EV-specific HPV types have been described in other immunosuppressed individuals such as renal transplant recipients (65). Patients with EV often have impaired cell-mediated immunity, which is believed to be important with regard to the manner in which they respond to infections by this subset of cutaneous HPVs.

About one third of patients with EV develop skin cancers in association with their lesions. Most of the malignant tumors remain local, but regional and distant metastases may occur. The risk of malignant progression is limited to the pityriasis-like lesions, which are the lesions that contain the EV types. HPV-5 and HPV-8 seem

to be the most oncogenic, since approximately 90% of the skin cancers contain one of these two types. The EV carcinomas usually arise in sun-exposed areas, suggesting that ultraviolet (UV) radiation may play a co-carcinogenic role with the specific HPVs in the etiology of these cancers. *p53* mutations are common in EV-associated cancer (66), in contrast to the mucosal cancers associated with HPV. Although metastasis is uncommon in the cancers in these patients, the presence of HPV-5 in the two metastatic lymph node lesions examined strengthens the agreement for an etiologic role for HPV in these carcinomas (67,68). Although the mechanistic role, if any, for these specific cutaneous HPVs in squamous cell cancers of the skin remains unclear, the E6 proteins have been shown to target Bak in UV-induced apoptosis (69).

Prevention and Therapy

A major advance has been the development of an effective preventive vaccine for the major genital tract HPVs. The vaccine is a subunit vaccine consisting of the major capsid protein (L1) that can self-assemble into viruslike particles (VLPs), which are empty capsids that closely resemble authentic virions morphologically and immunologically (70). The L1 VLPs are highly immunogenic, inducing high titers of neutralizing antibodies that are conformationally dependent and type-specific. Both Merck and GlaxoSmithKline have developed HPV VLP-based vaccines, both of which performed well in preclinical and proof of principle efficacy trials that reported almost complete protection in fully vaccinated women (three intramuscular doses, given over 6 months) against persistent infection or dysplasia attributable to the HPV type(s) targeted by the vaccine (71,72). High-level protection has now been shown to be durable for both vaccines, with serum antibody levels at least an order of magnitude higher than those seen following natural infection. Protection appears to be predominantly type-specific. In 2006, the U.S. Food and Drug Administration (FDA) approved the Merck vaccine—a quadravalent vaccine consisting of VLPs from HPV-16, HPV-18, HPV-6, and HPV-11, formulated in an alum adjuvant. GlaxoSmithKline has initiated phase 3 trials of its commercial vaccine, which is bivalent, composed of HPV-16 and HPV-18 VLPs in a proprietary adjuvant, AS04, which consists of alum plus monophosphoryl lipid A (MPL). One can anticipate that second-generation VLP vaccines may protect against an even higher proportion of HPV infection by incorporating VLPs from a larger number of HPV types. Although 70% of cervical cancers are caused by HPV-16 or HPV-18, 30% are caused by other high-risk HPV types.

There are several important unresolved issues for the current VLP vaccines (70). These include how long protection will last and if booster vaccination will be necessary, whether the vaccine has any therapeutic efficacy or can prevent the spread of infection to new sites, whether vaccination will be recommended for males before efficacy data in males are available, and whether there is any cross-protection afforded against HPV types not present in the vaccine. Furthermore, the VLP vaccine is expensive and is not heat stable, two characteristics that might impede its use in developing countries where the cervical cancer disease burden is greatest.

The impact of the vaccines on the reduction in the number of serious infections attributable to the HPV types in the vaccines

will be seen much sooner than the impact on the incidence of cervical cancer, which may take 20 years or more. Because of the type specificity, the current vaccines are unlikely to protect against a substantial proportion of other high-risk HPV type infections, it will be important for vaccinated women to continue to undergo cervical cancer screening.

Additional approaches to improve the vaccine seem warranted. The use of L2 represents a potential alternate approach to develop a prophylactic vaccine against a broader spectrum of HPV types. Although they are not as immunogenic as the L1 neutralization epitopes, at least some of the L2 neutralization epitopes induce cross-neutralizing antibodies against PVs from different types (73,74). In addition, modifications of the L1 capsid protein allow the self-assembly of capsomeres that are highly immunoprotective, can be produced in bacteria, and are more stable (75).

Epstein Barr Virus

Epstein Barr virus (EBV) is a common virus with a worldwide distribution. More than 90% of individuals worldwide have been infected by the time they reach adulthood. EBV was discovered from studies of a lymphoma described in young children in certain parts of East Africa. Although this childhood lymphoma had been previously recognized, it was first clearly defined as a unique entity with characteristic clinical, pathologic, and epidemiologic features by Dennis Burkitt in 1958 (76,77). In his early descriptive studies, it was speculated that the lymphoma could be due to a virus because its geographic distribution in a belt across equatorial Africa was similar to that of Yellow fever. In 1964, Epstein and Barr described virus particles of the herpesvirus family in lymphoblastoid cells from patients with Burkitt lymphoma (BL)(78,79). The finding of such virus particles in lymphoid lines, however, was not restricted to tissues from patients with BL, because these particles could also be observed in cell lines established from patients with other malignancies, from patients with infectious mononucleosis, and even occasionally from healthy individuals. Nonetheless, EBV was the first virus to be recognized as a human tumor virus.

Virus-Host Cell Interactions

EBV is a double-stranded DNA virus and is a member of the herpesvirus family. Other members of the human herpesvirus family include herpes simplex viruses types 1 and 2, varicella zoster virus, the cytomegalovirus, the human herpes virus types 6 and 7, and the Kaposi sarcoma herpesvirus (KSHV, also known as HSV-8). The mature EBV particle is essentially indistinguishable from those of the other herpesviruses. Herpesviruses are large viruses, measure 150 to 180 nm in diameter, and contain a large double-stranded DNA genome of about 170,000 bp of genetic information. In addition to this central core of genetic material, the virus particle consists of a capsid layer made up of capsomeres in an icosahedral shape and an outer lipoprotein envelope. EBV is considered a member of the γ herpesviruses because of its tropism for lymphoid cells, both in vivo and in vitro. EBV infects epithelial cells of the oropharynx and B-lymphocytes. The infection of B cells is a latent infection in which there is no replication of the virus and the cells are not killed. EBV proteins are serologically distinct from proteins

of other human herpes viruses. Early sero-epidemiologic studies established that antibody to EBV is prevalent in all human populations and that high titers of antibody correlate with infectious mononucleosis (80) and specific malignancies: BL, nasopharyngeal carcinoma (NPC), and Hodgkin disease (81). EBV is also associated with B-cell lymphomas in immunosuppressed individuals, particularly those with HIV or organ transplant recipients.

EBV has several distinct programs of gene expression in infected cells, a lytic cycle, and a latent cycle. The lytic cycle results from the phased expression of viral proteins that ultimately results in the replication of the virus and the production of infectious virions. The replicative cycle of EBV does not inevitably result in the lysis of the infected host cell because EBV virions are produced by budding from the infected cell. Latent infections do not result in the production of progeny virions. A limited number are produced during latent cycle infection. In latently infected B-lymphocytes, the genome circularizes an episome in the cell nucleus. In B-lymphoid cells that harbor and express the EBV genome in a latent state, there is expression of a distinct subset of viral proteins, including the EBV-induced nuclear antigens (EBNAs), including EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-leader protein (EBNA-LP). In addition, EBV encodes two latent infection-associated membrane proteins (LMPs) and two small nonpolyadenylated RNAs (EBERs) that are also expressed in EBV latently infected cells. Molecular genetic analyses using specifically mutated EBV recombinants have revealed that EBNA-3B, LMP2, the EBERs, and most of the viral genome that is expressed in lytic infection can be mutated without a significant effect on the ability of the virus to transform primary B-lymphocytes (81). The other EBNAs and LMP1 are important for lymphocyte transformation. LMP2 is important in maintaining latency by preventing lytic infection in response to lymphocyte-activation signals. A detailed description of the molecular biology of EBV and its normal biology is described in *Fields Virology* (81,82).

Two of these genes, *EBNA2* and *LMP1*, are particularly important with regard to viral latency and EBV immortalization of human B cells. EBNA-1 is a DNA binding protein that binds to an EBV origin of DNA replication called oriP and mediates genome replication and partitioning during division of the latently infected cells. EBNA-1 also possesses a glycine-alanine repeat that functions to impair antigen processing and major histocompatibility complex (MHC) class I-restricted antigen presentation of EBNA-1 thereby inhibiting the CD8-restricted cytotoxic T-cell recognition of virus-infected cells.

LMP1 has been shown to alter the effect of the growth properties of rodent cells, epithelial cells, and B-lymphocytes. LMP1 is a transmembrane protein that is essential for EBV-mediated growth transformation. LMP1 mediates signaling through the tumor necrosis factor- α (TNF- α)/CD40 pathway. When expressed in normal resting B-lymphocytes or EBV-negative lymphoblastoid cell lines, LMP1 induces most B-lymphocyte activation and adhesion markers, activates NF- κ B, and induces Bcl2 and A20, proteins important in preventing apoptosis. The C-terminal LMP1 cytoplasmic domain interacts with cellular proteins that transduce signals from the tumor necrosis factor receptor (TNFR) family. TNF signaling is critical in normal lymphoid development and the

B-lymphocyte TNFR family member, CD40, is remarkably similar in its growth-promoting and NF- κ B activating-effects to LMP1. The evidence supports a model that LMP1 mimics a constitutively activated TNFR (82).

EBNA-2 is the main viral transcriptional transactivator that has effects on viral and cellular genes. Viral proteins whose expression can be increased by EBNA-2 include the latent membrane protein (LMP1) and another membrane protein that is expressed in latently infected cells called terminal protein. EBNA-2 lacks DNA sequence-specific binding activity and is dependent on interactions with sequence-specific cell proteins for recognition of enhancer elements. EBNA2 binds the cellular RBPJ protein and is recruited to promoters regulated by RBPJ and leads to the constitutive activation of the Notch pathway. EBNA3A, and 3C also regulate transcription in lymphocyte transformation and, like EBNA-2, EBNA-3A, and EBNA-3C, also achieve specificity in their interaction with viral and cellular promoters by interacting with the cell protein RBPJ κ . Through the interactions of EBNA-2, EBNA-3A, and EBNA-3C with the cell protein, J κ , EBV therefore affects the cellular Notch signaling pathway (82).

Burkitt Lymphoma

In Africa, BL occurs several years after the primary infection with EBV. BL is a monoclonal lymphoma (83), as opposed to infectious mononucleosis, which is a polyclonal disease caused by EBV. African BL is clinically characterized by rapid growth of the tumor at nonlymphoid sites such as the jaw or the retroperitoneum. The tumor is of B-cell origin and is morphologically similar to the small, noncleaved cells of normal lymphoid follicles (84). The biopsy specimens from African BL invariably contain the EBV genome and are positive for EBNA (85). This is in contrast with the non-African BL in which only 15% to 20% of the tumors contain the EBV genome. The clustering of BL in the equatorial belt of East Africa cannot be explained solely on basis of EBV infection because it is found in all human populations. Potential effects on the immune system, possibly due to hyperstimulation by endemic malaria, have been postulated to play an important role in the outcome of an EBV infection in this region of Africa. Individuals from this region do have impairment of virus-specific cytotoxic T-cell activity, and it is the T-cell response to EBV infection that limits B-cell proliferation due to EBV stimulation (86). The failure of the T-cell immune response to control this proliferation might be an early step providing the enhanced opportunity for further mutation, oncogenic transformation, and lymphomagenesis in the actively dividing B-cell population. BLs often contain chromosomal abnormalities in regions that contain the immunoglobulin genes, most notably chromosomes 2, 14, and 22. In greater than 90% of BL, a translocation of the long arm of chromosome 14 (containing the heavy-chain immunoglobulin genes) to chromosome 8 (containing the *c-myc* oncogene) is observed (87). Less frequent translocations involve chromosome 2 (κ light chain) and chromosome 22 (λ light chain) (88). These translocations to chromosomes 2 and 22 generally involve reciprocal translocations to the distal arm of chromosome 8, containing *c-myc*. The expression of the *c-myc* oncogene following this translocation is deregulated due to the proximity of the *c-myc* oncogene to the

transcriptional control elements of the immunoglobulin genes that are active in B cells. Overexpression of the *c-myc* oncogene itself is not sufficient for malignant transformation of a B cell. Additional mutations can then occur in these B cells, leading eventually to the emergence of a monoclonal B-cell neoplasm. As such, EBV does not act directly as an oncogene, but rather indirectly as a polyclonal B-cell mitogen, setting the stage for the translocation to activate the *c-myc* oncogene and other mutations.

What is the role of specific EBV genes in the maintenance of BL? As noted previously, EBNA-2 and LMP1 appear to be the mediators of EBV-induced growth effects in B-lymphocytes. However, these are not expressed in BL and thus are not required for BL growth. It is possible that altered *myc* expression may replace the need for EBV oncogenic functions. Furthermore the down-modulation of the EBV EBNA and LMP functions may actually be advantageous to tumor development allowing the cell to escape from T-cell-mediated immune surveillance. It has been shown that the EBNA-2 and LMP1 can serve as targets of immune cytotoxic T cells, and that LMP1 induction of cell adhesion molecules can enhance the HLA-restricted killing of EBV-infected T cells (89).

Nasopharyngeal Carcinoma

NPC is also linked to EBV. NPC occurs in adults from ages 20 to 50, although in certain parts of Africa the age distribution extends to children as well. In general, males outnumber females 2 to 1, and although worldwide the annual incidence rates are low, there are areas in China (especially the southern province) in which there is a high rate of approximately 10 cases per 100,000 per year. Since the incidence among individuals of Chinese descent remains high, regardless of where they live, a genetic susceptibility has been proposed. Environmental factors have been implicated as risk factors for NPC, including fumes, chemicals, smoke, and ingestion of salt-cured fish that contain nitrosamines.

EBV genomes are found in nearly all biopsies of undifferentiated NPC specimens from all over the world (90,91). The genome is present in the epithelial cells of the tumors and it is noteworthy that all forms of NPC contain clonal EBV episomes, suggesting that the tumors arise from a single infected cell (92). The EBV genome is transcriptionally active within these tumors and the regions that are transcribed in the NPC biopsies are the same as those expressed in latently infected lymphocytes (93). These molecular observations are consistent with an active role for EBV in the neoplastic processes involved in NPC. Patients with NPC have elevated levels of immunoglobulin G (IgG) antibodies to EBV capsid and early antigens. Patients with NPC have serum IgA antibodies to capsid and early antigen, likely reflecting the local production of such antibodies in the nasopharynx. Cytogenetic studies on NPC xenografts have identified abnormal markers on a number of different chromosomes. Loss of heterozygosity has been noted by studies using restriction fragment length polymorphism (RFLP) on two different regions of chromosome 3, mapping to 3p25 and 3q14 in a very high percentage of NPC specimens.

The presence of immunoglobulin markers for EBV (IgA/VCA and IgA/EA) has provided the opportunity for early serologic identification of patients with NPC. The frequency of IgA

antibody to the EBV capsid antigen of 150,000 Chinese studied was found to be 1%. About 20% of the patients with elevated IgA antibodies to VCA had NPC, however, when biopsied. Thus, early detection using serologic tests can be applied in areas where NPC is prevalent, possibly leading to early therapeutic intervention.

EBV-Associated Malignancies in Immunocompromised Individuals

Perhaps the best evidence for the oncogenic potential for EBV comes from its association with a variety of malignancies in immunocompromised individuals. These include EBV-positive lymphoproliferative disease in children with primary immunodeficiencies affecting T-cell competence (such as those with the Wiskott-Aldrich syndrome or post-transplant lymphoproliferative disease (PTLD)), B-cell lymphomas in patients with AIDS, and smooth-muscle cell tumors of the immunocompromised patient (81).

EBV is associated with B-cell lymphomas in patients with acquired or congenital immunodeficiencies and in organ transplant recipients. These lymphomas can be distinguished from the classic BLs in that the tumors are often polyclonal. Also, the tumors do not demonstrate the characteristic chromosomal abnormalities of BL described in the preceding sections. The pathogenesis of these lymphomas involves a deficiency in the effector mechanisms needed to control EBV-transformed cells.

The association between EBV and leiomyomas and leiomyosarcomas in immunocompromised patients was unexpected and has now been seen in the context of acquired immunodeficiency and in organ transplant recipients. From the EBV standpoint, the pathogenesis and the role of EBV is not yet well understood (81).

Hodgkin Lymphoma

Serologic and epidemiologic studies have suggested a possible link between Hodgkin lymphoma (HL) and EBV; however, the high EBV infection rate in humans has made interpretation of these data difficult. These sero-epidemiologic studies are supported by molecular studies demonstrating EBV DNA, RNA, and proteins in HL pathologic specimens (94–97).

Generally, 60% to 90% of cases of mixed cellularity (MC) and lymphocyte-depleted (LD) subtypes of HL are EBV positive by *in situ* hybridization for EBER RNA or staining for LMP1, staining whereas only 20% to 40% of the nodular-sclerosing (NS) subtype are positive. Interestingly, in a given EBV-positive specimen, it is usually the large binucleate Reed-Sternberg cells and the mononuclear variant Hodgkin cells that are EBV positive. The nonmalignant cells in the tissues do not generally contain demonstrable levels of EBV DNA, RNA, or protein. Furthermore, expression of LMP1 in the Reed-Sternberg and Hodgkin cells can be demonstrated in a large percentage of the EBV-positive cases of MC and NS subtypes, although EBNA-2 expression is not detected (96,98). Thus, EBV infection may be one of several steps involved in Hodgkin disease. A clearer understanding of the molecular pathogenesis of HD is necessary to interpret the specific role EBV may play in its etiology. The evidence that EBV, when present, plays a causative role in the pathogenesis of HL is strong, but still circumstantial (81).

Viruses and Liver Cancer

Hepatocellular carcinoma (HCC) is one of the world's commonest malignancies. In China alone, there are between 500,000 and 1,000,000 cases of HCC per year. HCC is etiologically linked to infections by two different types of viruses, hepatitis B virus (HBV) and hepatitis C virus (HCV). Though relatively rare in the West, HCC is quite prevalent in Southeast Asia and in sub-Saharan Africa. In the 1970s, this distribution was recognized to mirror the distribution of chronic HBV infection. Indeed the long-recognized association between HCC and chronic hepatitis led to the strong presumption that chronic HBV infection predisposes to hepatic cancer. This presumption was validated in large, prospective, epidemiologic studies in Taiwan in which chronic infection with HBV leading to cirrhosis was shown to be of major importance in the etiology of this tumor (99). Chronic HBV infection was found to be associated with about a 19-fold increase of HCC mortality risk in men and a 33.5-fold increase in women (100). The World Health Organization has estimated that 80% of HCC worldwide occurs in individuals who are chronically infected by HBV. For the remaining 20% of HCC not associated with HBV, there are a number of additional risk factors including chronic hepatitis associated with HCV. Between 30% and 70% of HBV-negative cases of HCC are seropositive for HCV. In the United States, it has been estimated that as many as 40% of the cases of HCC are due to HCV.

Hepatitis B Virus (Virus–Host Interactions)

HBV is a member of a group of animal viruses referred to as the hepadnaviruses (for hepatotropic DNA viruses). HBV is the only human member of this group of viruses. Other members of this group include the woodchuck hepatitis virus (WHV), the Beechey ground squirrel hepatitis virus (GSHV), the Pekin duck hepatitis B virus (DHBV), and the gray heron hepatitis virus. These viruses share a similar structure and each is hepatotropic, leading to persistent viral infections of the liver. The animal hepatitis viruses have been very important contributors to our understanding of the molecular biology of these viruses. Of the hepadnaviruses, only HBV and WHV have been associated with chronic active hepatitis and HCC.

The reader is referred to a chapter on the molecular biology of the hepadnaviruses for details on the virus and aspects of virus/host cell interactions (101). Hepatitis B viral particles contain small, circular DNA molecules that are only partially double-stranded. The DNA consists of a long strand with a constant length of 3,220 bases and a shorter strand that varies in length from 1,700 to 2,800 bases in different molecules. A map of the HBV DNA genome is shown in Figure 6-4. The virion particles contain a DNA polymerase activity that is capable of repairing the single-stranded DNA region to make two fully double-stranded molecules, each approximately 3,220 bases in length. For this reaction, DNA synthesis initiates at the 3' end of the short strand that, as noted previously, is heterogeneous among different DNA molecules. DNA synthesis terminates at the uniquely located 5' end of the short strand when it is reached. The long strand is not a closed molecule but contains a nick at a unique site approximately 300 bp from the 5' end of the short strand.

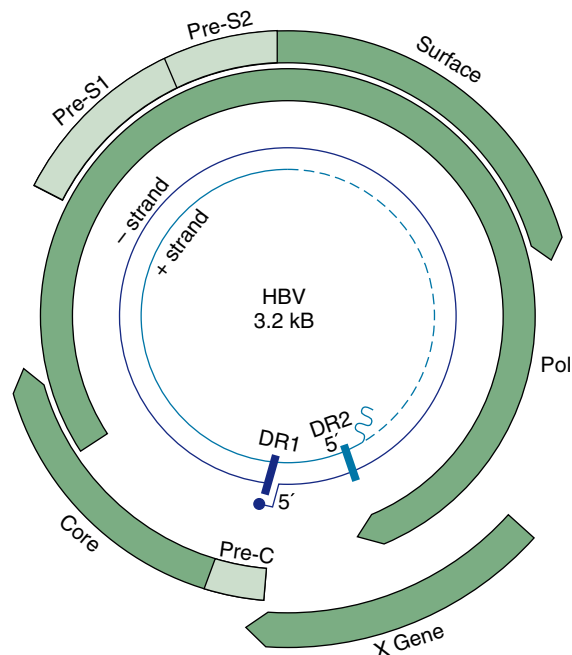


FIGURE 6-4 MAP OF THE HEPATITIS B VIRUS (HBV) GENOME. The arrows surrounding the genome represent the four large open reading frames of the L (–) strand with the genes they encode indicated. The broken line is the S (+) DNA strand. The positions of the 5' ends of the DNA strands are indicated. The locations of the direct repeats (DR1 and DR2) involved in the initiation of DNA replication are also indicated.

The HBV genome has four ORFs and encodes four genes. These ORFs are designated as S and pre-S, C, P, and X. S and pre-S represent two contiguous reading frames and code for the viral surface glycoproteins. C contains the coding sequences for the core structural protein of the nucleocapsid. The P gene encodes the viral polymerase that contains reverse transcriptase activity. The X ORF encodes a basic polypeptide that has transcriptional trans-activation properties that can up-regulate the activity of hepadnavirus promoters. The overall structure of the genomes of all of the animal hepadnaviruses is quite similar. The WHV and GSHV genomes are approximately 3,300 bp in size, and the DHBV is approximately 3,000 bp in size. The genomic organization of the different hepadnaviruses is similar and there is extensive nucleotide homology between them. The mammalian hepadnaviruses differ from the avian hepadnaviruses in that the avian hepadnaviruses do not contain the X region.

HBV DNA can be found free or integrated in the host chromosome of the hepatocyte. Free HBV DNA represents intermediate forms of replication for the viral genome and can be detected during acute infections and some chronic stages of HPV infection. Integrated sequences are usually found during chronic virus infection and in HCC. The replication mechanism for the hepadnaviruses, first discovered by Summers and Mason for DHBV (102) and later confirmed for HBV, is different from that of other DNA viruses (103). The replication cycle involves a reverse transcription step resembling that of the retroviruses in that it goes through an RNA copy of the genome as an intermediate in replication. The hepadnaviruses differ from the retroviruses, however, in that retrovirus virions contain RNA and the intermediate form

of replication is integrated DNA. The virions of the hepadnaviruses contain DNA and the intermediate replication form is RNA. Integration of the hepadnavirus genome as a provirus is not a necessary intermediate step for viral genome replication as it is for a retrovirus. The similarity between the retroviruses and the hepadnaviruses extends to the overall genomic organization in which all of the genes are encoded on only one strand. The order of the genes within the retroviruses (gag, pol, and env) is similar to their counterparts for the hepadnaviruses (core, polymerase, and surface antigen). Other subtle differences in the transcriptional programs used to generate the messenger RNAs for these different viruses exist. A further similarity between these viruses is that some members of each group of these viruses encode transcriptional regulatory factors. For HTLV-1, described earlier in this chapter, the X region encodes the transcriptional activator tax as well as the rex gene product involved in messenger RNA transport to the cytoplasm. The X genes encoded by the mammalian hepadnaviruses similarly encode a protein that has been extensively studied and shown to have a variety of activities, including the ability to function as a transcriptional activator. However, the function of X in the life cycle of the mammalian hepadnaviruses is not well understood. Although there have been studies claiming that the X protein has oncogenic properties, the evidence implicating a direct role for the X protein in HCC is far from compelling (104).

Primary infection with HBV results in a subclinical infection or acute hepatitis B, depending on the age of the individual, among other factors. In adults, 95% of such infections resolve, with clearance of virus from the liver and the blood with lasting immunity to reinfection. The remaining 5% of infections do not resolve, but develop into a persistent hepatitis with a viremia that usually last for the life of the host and can have a variety of pathologic consequences. Many of these persistent infections are with little hepatocellular injury. Approximately 20% to 25% of persistently infected individuals develop hepatocellular injury: chronic persistent hepatitis (in which case the inflammation is limited to periportal areas) or chronic active hepatitis (where there is inflammation and hepatocellular necrosis extending outside of the portal areas). Chronic active hepatitis has significant potential for progression to cirrhosis, hepatic failure, and cancer.

HBV and Hepatocellular Carcinoma

An etiologic role of HBV in human HCC is now established. There is a striking correlation between the worldwide geographic incidence of HCC and the prevalence of HBsAg chronic carriers, and important evidence for the role of HBV in HCC was provided by the prospective epidemiologic studies of Palmer Beasley in Taiwan (99). The classic studies from Beasley demonstrated that chronic HBV carriers in Taiwan had a more than 100-fold risk over noncarriers for the development of HCC. In areas endemic for HBV like Taiwan, infection with the virus occurs in early childhood, and there is an interval of approximately 30 years before the development of HCC.

Despite the strong epidemiologic evidence that establishes HBV as the major cause of HCC worldwide, a mechanistic role for HBV in HCC is not fully understood. Usually in HBV-positive

liver cancers, viral DNA sequences can be found integrated into the host cellular DNA. Different tumors display different patterns of integration, indicating that the insertion of the viral DNA into the host chromosome is not site specific. In a given tumor, however, all cells have the same pattern of HBV DNA integration, indicating that the integration event preceded the clonal expansion of the tumor. This clonal pattern of HBV DNA integration supports the etiologic role of HBV in HCC. The HBV-integrated genomes are often highly rearranged within tumors, displaying a variety of deletions, inversions, and point mutations. Although occasional integrated genomes retain one or more viral genes intact, there does not appear to be a consistent pattern in which one gene is regularly preserved intact. This indicates that the continued expression of a specific viral gene is not required for the maintenance of the malignant phenotype in an HBV-positive liver cancer.

Thus, the major question is: How does HBV cause HCC? There are two hypotheses that have emerged, one involving a direct role of the virus in carcinogenesis and the other indirect, as a consequence of persistent liver injury caused by the immune response to infected hepatocytes.

The direct models implicate an oncogenic role for an HBV either through the integration of the viral genome or from the oncogenic activity of a viral gene product. The indirect models do not require a direct genetic contribution by the virus or its gene products to the transforming event (104).

Mechanisms by which HBV DNA integration could directly contribute to tumorigenesis could be (1) proto-oncogene activation as a result of the insertion of the viral DNA or (2) the inactivation of tumor-suppressor alleles by such integration. Indeed, there is compelling evidence that insertional activation in WHV-induced hepatomas is important in hepatocellular carcinogenesis in the woodchuck model. Approximately 20% of the tumors show WHV DNA inserted into the N-myc locus (105). This gene, normally silent in adult liver, is strongly up-regulated by this insertion, and this activation can be seen early in the oncogenic sequence—even in premalignant lesions. Whereas insertional activation of N-myc clearly plays a major role in WHV oncogenesis, a similar claim cannot be made for HBV. Human hepatomas do not harbor N-myc rearrangements. An extensive search for comparable events in HBV-associated human HCC has revealed only rare examples of integration in loci that might contribute to tumorigenesis described (i.e., insertions near loci for retinoid receptors, erb-A or cyclin A; 99). In conclusion, although insertional mutagenesis or specific oncogene activation may be important in individual cases of HCC, there is little evidence that it is of general mechanistic importance for HCC in humans.

There is also no strong evidence that HBV encodes a transforming protein. The best candidate may be the viral X protein, a small regulatory protein that is encoded by the oncogenic mammalian hepadnaviruses but not by the nononcogenic avian hepadnaviruses. Indeed, transgenic mice with high levels of hepatic expression of X develop hepatocellular carcinoma with increased frequency (106). Tumors in these mice do not begin until midlife, suggesting that additional genetic changes are necessary for cancer development. The X protein has no homology to known oncogenes or cellular genes involved in signaling or growth control.

X has been extensively studied from a functional standpoint, but its precise role in the hepadnavirus life cycle remains unclear. The X protein can stimulate cytoplasmic signal transduction pathways (e.g., the ras-raf MAP kinase pathway)(107,108), function as a nuclear transcriptional activator (109,110), and interfere with cellular DNA repair by binding DNA repair proteins (111). The relationship of all of these activities to the putative oncogenic function of X is unproven. It may be that the role of the HBV X gene product in tumorigenesis in the transgenic mouse lines that have been derived is an indirect one, possibly due to liver injury and triggering hepatocellular regeneration from the overexpression of the X protein. It is also important to remember that X is not always expressed in HBV-positive HCCs.

In the absence of strong data to support a direct oncogenic role for HBV in HCC pathogenesis, there is mounting support for a more indirect model, in which neither the HBV genome nor any of its products make a direct genetic contribution to the transformation of the infected cell. Instead, HBV-induced cellular injury, a consequence of the immune or inflammatory responses to HBV infection, results in liver cell regeneration that, over time, can lead to cancer. Cellular proliferation in liver regeneration increases the chances for errors in DNA replication leading to mutations that can contribute to the loss of normal cellular growth control. Those cells with an appropriate set of genetic mutations can then undergo clonal expansion and ultimately progress to HCC. In this indirect model, HBV promotes oncogenesis chiefly by provoking cellular proliferation in response to immune-mediated injury. As such, no direct genetic contribution is made by viral sequences acting in *cis* or viral gene products acting in *trans*. Significant experimental evidence for the indirect model for HBV-induced carcinogenesis has come from the important experiments using HBV in transgenic mice (112–114).

Although the tumorigenic mechanisms of HBV-induced carcinogenesis remain unclear, the overwhelming epidemiologic data clearly establish HBV as the principle cause of most cases of HCC worldwide. Thus, an effective vaccine to prevent HBV infection would be expected to prevent most cases of HCC. A vaccine consisting of HBsAg produced by recombinant techniques in yeast or CHO cells in tissue culture is highly immunogenic and can protect against HBV infection (115). Remarkably, the vaccine is quite effective in newborn infants, in whom vaccines are often poorly immunogenic. In geographic areas of high virus prevalence, the goal of universal vaccination of infants is to prevent infections that result in viral persistence in the population, and thus prevent HCC. Vaccination programs are now under way. A reduction in the levels of HCC following a reduction in the HBV carrier rates among the vaccinated populations will provide confirmation of the role of HBV in HCC. Data from a universal vaccination program in Taiwan have already indicated that HBV vaccination may reduce the number of rare childhood cases of HCC (116).

Hepatitis C Virus

Hepatitis C virus (HCV) is a human flavivirus, a positive-strand RNA virus. HCV is an important cause of morbidity and mortality worldwide. HCV carries a high rate of chronicity

after infection, with over 70% of those infected going on to develop chronic liver disease. HCV is believed to be the leading infectious cause of chronic liver disease in the Western world. HCV is also etiologically responsible for many cases of HCC worldwide. Between 30% and 70% of HBV-negative HCC patients are seropositive for HCV. In the United States, it appears that as many as 40% of the cases of HCC may be associated with HCV. HCV positivity conveys about an 11.5-fold increased risk for the development of liver cancer (117).

HCV was first cloned in 1989 from the infectious sera of individuals with post-transfusion hepatitis (118,119). Much of our knowledge of this virus derives from molecular genetic and biochemical studies because, until recently, there were no suitable tissue culture systems or animal models for the study of this virus. Nonetheless, there have been major advances in our understanding of the molecular biology of this important human pathogen (120). HCV is a single, positive-stranded RNA virus with a 9.4-kb RNA genome that contains a single ORF encoding a polypeptide of 3,011 amino acids. This large polypeptide is then post-translationally cleaved to produce several mature structural and nonstructural proteins. The HCV virus is inherently unstable, giving rise to multiple types and subtypes. This genome instability is due to the dependence of the virus on the virally encoded, RNA-dependent RNA polymerase to perform the RNA-to-RNA copying of the genome. There is no DNA intermediate in the replication of the genome, excluding the possibility of viral genome integration as a mechanism for HCV-associated carcinogenesis. Furthermore, the polymerase lacks proofreading capability, and there is a substantial level of base misincorporation, accounting for the marked heterogeneity in viral isolates, even from a single infected individual. There is a high degree of variability in the viral envelope glycoproteins, which has led to the hypothesis that changes in these genes alter the antigenicity of the virus over time, permitting the virus to escape immune recognition by the host. This variability allowing the virus to escape the immune system is important to the pathogenesis of the virus in establishing a persistent infection. A characteristic feature of an HCV infection is repeated episodes of hepatic damage, resulting from the reemergence of a newly mutated genotype. This genomic heterogeneity, due to the ability of the virus to rapidly mutate, has proved problematic to attempts to develop an effective vaccine to HCV.

It is unclear whether HCV contributes directly to hepatocarcinogenesis. As noted previously, HCC does not replicate through a DNA intermediate, and therefore cannot integrate into host chromosomes causing insertional mutagenesis. The virus encodes several nonstructural (NS) proteins that are involved in viral genome replication and in altering the cell environment to allow a persistent infection. For instance one of the nonstructural proteins, NS5A, can affect interferon signaling and cellular apoptosis through interactions with specific cellular proteins. Interactions of some of the HCV NS proteins with cellular proteins involved in cellular tumor suppression pathways have been described and a few reports suggest some oncogenic properties for the viral NS proteins in transfection experiments (120). However, there is no compelling body of evidence that would suggest HCV encodes a protein that directly contributes to HCC development. Instead,

as with HBV, most data suggest that the role of HCV in hepatocarcinogenesis is indirect through persistent infection, chronic inflammation, and cirrhosis.

Kaposi Sarcoma Herpesvirus

KSHV, also known as human herpesvirus 8 (HSV-8), is a γ -2 herpesvirus. Chang and Moore discovered this virus in 1994 by representational difference analysis in an AIDS Kaposi sarcoma (KS) skin lesion (121). Since its discovery, KSHV has been linked with several other different tumors in addition to KS: body cavity-based or primary effusion lymphomas (PEL) and some plasma cell forms of multicentric Castleman disease (MCD).

KS was initially described as an aggressive tumor by Moritz Kaposi in the nineteenth century. Before the onset of the AIDS epidemic, KS had been described as a rare and indolent tumor of elderly Mediterranean men and was later recognized to occur more frequently in parts of Africa. KS had also been observed among immunosuppressed organ transplant recipients. KS is the most common neoplasm associated with the acquired immune deficiency syndrome (AIDS) (122,123). The histology of KS in all of these clinical settings is similar. KS lesions contain multiple cell types, including spindle cells, which are believed to arise from an endothelial, cell precursor, and infiltrating mononuclear cells. KS lesions are histologically characterized by slitlike vascular channels that give the lesions their distinctive reddish clinical appearance.

The discovery of KSHV by Chang and Moore was a major advance for our understanding of the etiology and pathogenesis of KS (121). It had been previously suspected that KS was associated with a virus, and indeed there had been a number of studies in the literature implicating a variety of other viruses, none of which stood the test of time. Using specific DNA fragments identified by representative differential analysis, DNA-based detection of viral sequences soon allowed the extension of the findings of Chang and her co-workers, to establish the association of KSHV with HIV-related and -unrelated forms of KS (124). The DNA was also found in PEL and MCD (125,126).

Research with PEL cell lines and on the KSHV itself quickly moved the field along and established an etiologic role for the virus in KS (127). The identification of KSHV-positive PEL cell lines (128,129) led to the development of some initial serologic assays for epidemiologic and virologic studies of the agent (124). Within 2 years of the first report of the agent, the full-length 165-kb genome of KSHV was sequenced from a PEL-derived cell line (130) and from a KS lesion (131).

KSHV is a member of the rhadinovirus (or γ -2) subfamily of the herpesviruses. It is the only known human rhadinovirus and is closely related to herpesvirus saimiri of squirrel monkeys. Humans are the only known host for KSHV. Unlike other human herpesviruses, infection by KSHV is not ubiquitous, and only a small percentage of humans in developed countries are serologically positive for the virus. Infection by KSHV is characterized by a prolonged viral and clinical latency that, like other herpesviruses, may be life-long. In a setting of immunosuppression or immunodeficiency, individuals infected with KSHV may then develop

KS or other KSHV-associated tumors, years after the primary infection. At this point, a role for KSHV has been established for KS, PEL, and MCD.

Despite the progress in the epidemiology and molecular biology associated with KSHV, our understanding of the virology and the mechanisms of pathogenesis and carcinogenesis associated with this virus is still at an early stage. The virus has been difficult to culture in the laboratory and much of our knowledge has been discerned from the analysis of the primary sequence by studying individually encoded genes. One characteristic of the rhadinovirus subfamily of the herpesviruses is the presence of recognizable variants of cellular genes that appear to have been captured into the viral genomes, a process that has been termed “molecular piracy.” These genes are believed to play important regulatory roles in the virus life cycle, in evading the cells host defenses, and in causing its associated pathology in the host (132). Among the KSHV regulatory genes are viral genes that resemble cellular cytokines, cellular chemokines, the cellular interferon regulatory factor (IRF-1), the cellular apoptosis factor (FLIP), a viral homologue of Bcl-2, a viral cyclin that is resistant to inhibition by cdk inhibitors, and a chemokine receptor, among others (133). In addition, many of the KSHV regulatory genes resemble EBV genes or target cellular pathways that are also targeted by other DNA tumor viruses, particularly EBV. Included among this group of genes is *LAMP*, which is similar to the EBV *LMPI* and *LMP2A* genes. Much of the effort in the field has been focused on these individual genes and their properties. It is beyond the scope of this chapter to go into detail about the molecular biology of KSHV and these particular studies. Instead, the reader is referred to the comprehensive chapter on the molecular biology of KSHV in *Fields Virology* (133).

Human Retroviruses and Cancer

Human T-Cell Leukemia Viruses

Although there were prior claims, the first substantiated reports of a human retrovirus were published in 1980 and 1981 by Robert Gallo and his colleagues (134,135) and soon after from Yoshida and his colleagues in Japan (136). These isolates were from human T-cell leukemia cell lines. The human T-cell leukemia virus type 1 (HTLV-1) is recognized as the etiologic agent of adult T-cell leukemia (ATL). A causal relationship between HTLV-1 and ATL was initially suggested by epidemiologic studies showing geographic clustering of ATL, a pattern that is consistent with an infectious agent. A second human retrovirus, referred to as HTLV-2, was initially isolated in 1982 from a cell line established from a patient with an unusual form of hairy cell leukemia (137). However, studies have not established an association of HTLV-2 with any human malignancies.

ATL was first described by Takatsuki and his colleagues in 1977 (138) before the virus was discovered and it is a malignancy of mature CD4-positive lymphocytes. It is endemic in parts of Japan, the Caribbean, and Africa. Clinically the tumor resembles mycosis fungoides and Sezary syndrome but is more aggressive than these other two syndromes, with a median survival from the

time of diagnosis of only 3 to 4 months. In addition to the skin involvement, it affects visceral organs, often with an associated hypercalcemia.

Serologic assays specific for HTLV-1 viral antigens revealed that virus infection is more widespread in the endemic areas than was the prevalence of ATL (139). An HTLV-1-infected individual has about a 3% lifetime risk of developing ATL. HTLV-1 infection is most marked in the Southernmost islands of Japan and the Caribbean. After Japan and the Caribbean, parts of Africa appear to have the next largest reservoirs of infection. The prevalence in the United States and in Europe is low in the general population, although it is quite high among intravenous drug abusers. A preleukemic disease in the form of a chronic lymphocytosis often precedes the development of acute leukemia or lymphoma. ATL usually occurs in early adulthood, and this is believed to be approximately 20 to 30 years after the initial infection in the subset of individuals who develop it.

HTLV-1 infection has been associated with a second clinical entity: HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chronic degenerative neurologic syndrome that primarily affects the spinal cord. Specific risk factors that may be important in determining the development of ATL or TSP in the HTLV-1-infected individual are not known. Childhood transmission is usually from the mother through breast milk and can result in ATL in the small percentage of patients as adults several decades later. The factors that contribute to disease progression in the few percentage of HTLV-1-infected individuals who will develop ATL are not known. Alternatively, HAM/TSP usually occurs in individuals through parenteral transmission by blood transfusion, intravenous drug use, or sexual transmission. It is generally believed that HAM/TSP is primarily the result of an autoimmune process against the central nervous system (CNS) somehow initiated by the viral infection.

Epidemiologic studies have shown that about 2% to 5% of individuals seropositive for HTLV-1 will develop ATL. The virus is transmitted from mother to infants through mother's milk and in adults is transmitted through sexual contact and contaminated blood. The latency period between the time of infection and the development of ATL can vary from a few years to as long as 40 years. Some evidence suggests that the virus's role in leukemogenesis may be direct in that the virus alone appears to be sufficient to initiate a series of events that may lead to leukemia independent of subsequent environmental factors.

Molecular studies suggest a possible direct role of HTLV-1 as an etiologic agent in ATL. In the life cycle of a retrovirus, the provirus (i.e., the double-stranded DNA copy of the viral RNA genome) becomes integrated into the cellular genome at random positions as part of the life cycle of the virus. In the leukemic cells of a patient with ATL, however, the viral sequences are found integrated in the same place in each cell, although the site of integration varies from leukemia to leukemia. This indicates that ATL is clonal, all of the leukemic cells must necessarily derive from a single cell, and the viral infection must have preceded the expansion of the tumor.

HTLV-1 is also a transforming virus capable of immortalizing normal human umbilical cord blood lymphocytes (T cells) in

vitro. The mechanism by which HTLV-1 induces leukemogenesis is different from that of the other chronic leukemia retroviruses studied in animals such as the avian leukosis virus or the murine leukemia virus. The combination of the clonality of the tumor cells and the random nature of the integration sites of the provirus from tumor to tumor are not characteristics of the animal leukemia viruses and indicate that HTLV-1 transforms by a different mechanism. Before the studies of HTLV-1 transformation, two mechanisms were known by which a retrovirus could induce malignancy. The first mechanism involved the transduction of an oncogene directly by the retrovirus. Indeed, the avian sarcoma virus studied by Peyton Rous is capable of inducing tumors in chickens because it has acquired extra nucleic acids from the cellular oncogene called *src*.

The second mechanism by which retroviruses were known to cause malignancies is exemplified by the slow-acting leukemogenic retroviruses such as the feline leukemia virus (FeLV) and the mouse leukemia virus (MuLV). These viruses do not contain oncogenes and induce leukemia in only a minority of the infected animals by the virus. There is also a long latency between the time of viral infection and the time of tumor formation, and the tumors are clonal. The mechanism of leukemogenesis by these slow-acting animal retroviruses differs from that of HTLV-1. Although the provirus integrates randomly into the cellular chromosomes in infected cells, for the slow-acting leukemogenic animal retroviruses, integration occurs preferentially in the vicinity of cellular proto-oncogenes in the tumors that develop. The provirus for these viruses must integrate into a region of the cellular genome in a manner that allows the regulatory sequences of the provirus to activate a nearby oncogene to stimulate cellular proliferation. This mechanism is called "promoter insertion" if the proviral long terminal repeat (LTR) acts as a promoter to initiate transcription of the proto-oncogene and "enhancer insertion" if the LTR acts as an enhancer to activate the expression of proto-oncogene. For the avian leukosis virus, the integration of the retrovirus occurs in the vicinity of the *c-myc* oncogene, resulting in the deregulation of its expression and resulting in cellular proliferation.

The fact that HTLV-1 provirus integration site varies from leukemia to leukemia is consistent with the HTLV-1 genome encoding a factor that is critical in the early stages of leukemogenesis. HTLV-1 and its relative HTLV-2 belong to a distinct group of retroviruses that has been referred to as trans-regulating retroviruses, which includes the bovine leukemia virus, the biology of which is actually quite similar to that of HTLV-1 and HTLV-2. This group of retroviruses differs from the chronic leukemia viruses and the acute leukemia viruses as depicted in Figure 6-5 by the fact that they contain additional genes at the 3' end of the genome. This region is called the X region and encodes trans-regulatory factors involved in transcriptional activation, translational control, and mRNA transport from the nucleus. Two unique regulatory genes, *tax* and *rex*, encoded by this region have been particularly well studied (140). The *tax* gene serves as a master key for activating transcription from the viral LTR, and the *rex* gene is involved in the transport of specific viral messenger RNA species from the nucleus to the cytoplasm.

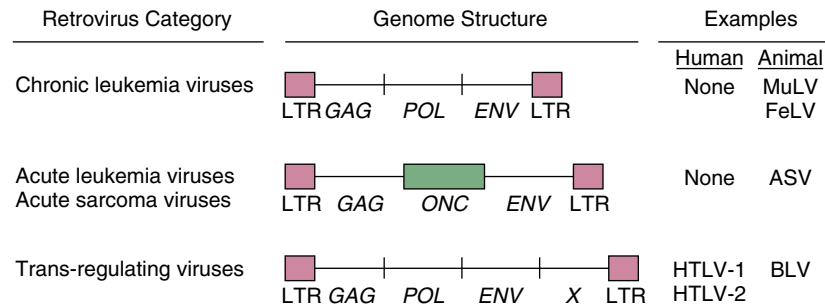


FIGURE 6-5 THE GENOMIC ORGANIZATION OF DIFFERENT TYPES OF RETROVIRUSES. The prototype retrovirus represented in the figure by the chronic leukemia viruses. It contains regulatory sequences at each end derived from the long terminal repeat (LTR) elements of the virus as well as the coding sequences for the viral proteins *gag*, *pol*, and *env*. The acute transforming retroviruses are defective viruses. Acquired onc sequences from the cellular genome replace critical viral gene segments. These defective viruses can therefore only replicate in the presence of a replication competent helper virus. The *trans*-regulatory retroviruses contain sequences, 3' to the *env* gene, which encode regulatory factors. This region has been referred to as the X region and encodes the *tax* and *rex* genes.

There is good evidence supporting a direct role for *tax* as a transforming gene in the causation of ATL. *Tax* can immortalize human CD4-positive T cells in an IL-2-independent manner, transform rodent fibroblasts in tissue culture, and induce tumors in transgenic mice (140). Multiple transforming activities of *tax* have been described that have been linked to its ability to activate specific cellular transcription factors, affect the cell cycle through interactions with cell-cycle-inhibitors, and inhibit apoptosis and cellular DNA repair.

Tax transactivates the viral LTR promoter through its interaction with CREB/ATF-1, CBP/p300, and the *Tax*-responsive 21-bp repeat element (TRE)(140). In addition, the *tax* gene product has been shown to activate transcription of specific cellular genes including lymphokines the IL-2 gene, and the IL-2 receptor gene through the NF- κ B pathway (141). HTLV-1 may initiate the leukemogenic process through activation of specific cellular genes by *tax*. One mechanism by which HTLV-1 could induce cellular proliferation and immortalization could involve an autocrine loop through the *tax*-mediated stimulation of IL-2 and its receptor. *Tax*-mediated activation of cellular genes may also involve paracrine mechanisms. *Tax* has also been shown to activate the expression of a group of nuclear oncogenes, including *c-fos*, *c-egr*, and *c-jun* (140). The mechanism by which *Tax* activates these various cellular promoters is through interactions with cellular transcription factors. The factors identified include CREB and the CRE modulator protein (CREM), the NF- κ B family of proteins, and the serum response factor (SRF).

Tax binds and inactivates the inhibitory proteins of NF- κ B called I κ B (142,143). There is a complex of I κ B proteins, most notably I κ B α , that binds and retains NF- κ B in the cytoplasm until there is a signal for activation, when I κ B α is targeted for proteolysis, releasing NF- κ B to translocate into the nucleus to activate transcription of its downstream effectors. Through binding I κ B, *Tax* destabilizes the I κ B/NF- κ B complex and activates NF- κ B. Thus the *Tax* mechanism of activation of genes under the control of NF- κ B appears to be two-pronged, first, through the suppression of its cytoplasmic tether, I κ B, and second through binding NF- κ B directly and bridging it with the basic transcriptional machinery.

In addition to its transcriptional activation functions, *Tax* affects many aspects of the cell cycle. *Tax* can complex with

p16INK4A, a cell-cycle inhibitor that binds and inhibits the activity of cell-cycle-dependent kinase 4 (*cdk4*)(144). *Cdk4* works with cyclin D to phosphorylate and inactivate the retinoblastoma protein (pRB). The consequence of *Tax* inactivation of p16INK4A is therefore the activation of cyclin D/*cdk4* and the inactivation of pRB, which in turn leads to cell-cycle activation, driving the proliferation of cells from G₁ to S. The pathway regulating pRB is commonly targeted by the DNA tumor virus oncoproteins as discussed earlier in this chapter. In addition, *Tax* is capable of inactivating *p53* functions (145), inducing p21^{CIP} expression (146), and inhibiting apoptosis and DNA repair (147). In addition *Tax* can dramatically perturb mitotic regulation, causing micronuclei formation, cytokinesis failure, and chromosome instability (148). These viral activities are likely important in the direct role of HTLV-1 in the initiation and progression of leukemogenesis.

HIV, AIDS, and Cancer

The human immunodeficiency viruses (HIV-1 and HIV-2) are members of a distinct subclass of retroviruses called lentiviruses (149). Similar to HTLV-1, the HIVs also infect CD4-positive T-lymphocytes. Beyond sharing a common cellular host for replication, however, the viruses are not closely related and do not share any serologic cross-reactivity. HIV-1 and HIV-2 are associated with AIDS. These viruses themselves do not appear to play a major direct etiologic role in any specific human tumors. Patients with AIDS, however, have a high incidence of specific tumors including KS and other cancers that are often caused by specific viruses (150). Indeed, one of the earliest diagnostic features of AIDS in homosexual males can be KS, a tumor that was regarded as extremely rare prior to the current AIDS epidemic. The etiology of KS involves KSHV (HSV-8) and is due to the uncontrolled proliferation of an activated microvascular endothelial cell, which is believed to be the cell of origin in KS (see section on KSHV).

Other tumors often seen in patients with AIDS include non-Hodgkin lymphomas and papillomavirus-associated cancers, including perianal squamous cell carcinomas and cervical cancer. Because of the immunodeficiency in AIDS patients, viral infections are common and some of the tumors seen in these patients likely have a viral etiology. For instance, a high percentage of AIDS

patients develop lymphomas, including CNS lymphomas. Some, but not all, of these lymphomas may be accounted for in part by the emergence of populations of B-lymphocytes transformed by EBV. It is also possible that HTLV-1 may account for some lymphomas in patients with AIDS. Patients with AIDS are often infected by PVs and HBV. The genital warts, anal and perianal squamous cell carcinomas, and cervical cancers seen in these patients are due to the specific HPV types that are oncogenic in immune-competent individuals.

SV40 and the Human Polyomaviruses

Periodic reports dating back to the 1970s claim the presence of SV40 DNA of SV40 in a variety of different human cancers, including osteosarcomas, mesotheliomas, pancreatic tumors, and brain tumors. This has been a controversial area and one that has recent scrutiny from investigators in the field and the National Cancer Institute. The studies are not summarized here; the reader is referred to a review of the subject (151,152).

SV40 is a nonhuman primate virus that naturally infects Asian macaques. The major source of human exposure to SV40 was through contaminated poliovirus vaccines that were given between 1955 and 1963. SV40 is a highly oncogenic virus in rodent cells and has served as an extremely valuable model for determining the various mechanisms by which DNA tumor viruses transform cells and contribute to tumor formation. However, there is no epidemiologic evidence indicating a higher risk of cancers among the populations of individuals who received the SV40-contaminated vaccine.

There is also no compelling data that the virus is circulating among human communities. SV40 is closely related to the human polyomaviruses BK and JC, and much of the seroreactivity to SV40 seen in humans can be accounted for by cross-reactivity with BK and/or JC virus. In addition, much of the data claiming an association of SV40 DNA with human tumors have been gathered by the use of the polymerase chain reaction (PCR) assays, which are error prone, and have been difficult to confirm. PCR primers used in many of these studies detected sequences that are present in many laboratory plasmid vectors, raising the possibility of laboratory contaminations. Some studies suggest that flawed PCR detection methodologies and laboratory plasmids could contribute significantly to the positive claims for SV40 tumor associations (153,154).

There have been periodic claims that the human polyomavirus BK and JC are also associated with specific human cancers. Infections with both of these viruses are widespread in humans as measured by seroreactivity. They encode tumor (T) antigens similar in function to SV40 large T-antigen and can functionally inactivate the p53 and pRB pathways. For JC virus, which is the cause of progressive multifocal leukoencephalopathy (PML), there have been reports of DNA and T-antigen in brain tumors of patients with or without PML. This is a provocative association that will need confirmation and validation. A number of studies have found an association of BK virus with a variety of different types of cancers as well as precancerous lesions of the prostate. The presence and potential role of these viruses in the cancers with which they have been found will need to be further explored.

Bacteria and Cancer

Helicobacter pylori and Gastric Cancer

Helicobacter pylori entered the scientific lexicon during the mid 1980s with the work of Robin Warren and Barry Marshall, who first cultured the bacterium and determined it was the causative agent of most gastric and duodenal ulcers (155,156). For their work, they shared the 2005 Nobel Prize in Medicine and upended the notion that gastric ulcers were mainly caused by stress and diet.

H. pylori (first known as *Campylobacter pyloridis*) is a gram-negative, flagellated spiral or curved bacilli that colonize the stomach via attachment to gastric epithelial cells. The complete genome was sequenced in 1997 (157) and predicted to encode for approximately 1,500 ORFs, many involved in adaptation for growth in the inhospitable acidic environment of the stomach. Infection is found in over 80% of the worldwide population, although a much smaller population develops gastric ulcers due to infection.

Two human cancers have been correlated with *H. pylori* infection: gastric cancer and MALT (mucosa-associated lymphoid tissue) lymphoma of the stomach. The correlation was strong enough to categorize *H. pylori* as a carcinogen by the International Agency for Research on Cancer (IARC). Gastric cancers are the fourth most common cancer worldwide. Since the isolation of the organism and the sequencing of its genome, a number of potential transformation mechanisms have been proposed, involving epithelia and immune cell populations. There are a number of possible mechanisms related *H. pylori*-induced transformation. One observation is that *H. pylori* produces excess free radicals, leading to host cell DNA damage and the accumulation of host cellular mutations.

A potentially oncogenic factor produced by *H. pylori* is the CagA protein. CagA is injected in gastric epithelial cells via type IV secretion and has been shown to alter a number of signal transduction pathways (158). One target of CagA is the SHP-2, a tyrosine phosphatase implicated in some human cancers. CagA induces SHP-2 activation, leading to disruptions in cell adhesion and cell junctions, and an increase in cell motility. Another factor produced by the bacteria is VacA, a secreted vacuolating cytotoxin protein that inhibits the ability of T-lymphocytes to neutralize infection and allows the bacterium to evade the immune system and set up a chronic infection. Another proposed mechanism of *H. pylori*-induced transformation has been called a "perigenetic pathway," which refers to effect of chronic inflammation has on host epithelial cells (159). Infection can induce TNF- α and IL-6, which can alter host cell adhesion and lead to migration of mutated cells.

Parasites and Cancer

Parasites were perhaps the first infectious agents to be potentially linked with human cancer. In 1900, Askanazy reported a link between *Opisthorchis felineus* infection with liver cancer, and Goebel published a report incriminating *Bilharzia* infections (schistosomiasis) with human bladder cancer (160). Indeed, the

Nobel Prize in Medicine was awarded to Johannes Fibiger in 1926 for studies linking a nematode with tumors in rats; however, those studies could not be reproduced. There are two, well-established associations of parasites with human cancer that will be presented: Shistosomiasis with bladder cancer and liver flukes with cholangiocarcinoma. The major burden for parasite-associated cancers is in developing countries.

Shistosomiasis and Bladder Cancer

Schistosomiasis, also known as bilharzia, is a parasitic disease caused by trematodes from the genus *Schistosoma*. *Schistosoma haematobium* is responsible for urinary schistosomiasis that can cause chronic infections that can lead to kidney damage and to bladder cancer. *S. haematobium* infections are a significant public health problem in much of Africa and the Middle East, second only to malaria among parasitic diseases. Bladder cancers associated with *S. haematobium* are squamous cell cancers and are histologically different from transitional cell carcinomas that are more commonly seen in the United States and Europe. The mechanism by which *S. haematobium* causes bladder cancer is unknown but most likely is a consequence of a persistent, chronic infection.

Liver Flukes and Cholangiocarcinoma

Opisthorchis viverrini and *Clonorchis sinensis* are liver flukes (a type of flatworm) that are associated with an increased risk of cholangiocarcinomas. Infections with these liver flukes come from eating raw or undercooked fish. They occur almost exclusively in East Asia and are rare in other parts of the world. Cholangiocarcinoma is more common in areas endemic to liver fluke infection (Hong Kong, Thailand). *O. viverrini* is endemic in northeast Thailand and is estimated to infect approximately 9 million people. *C. sinensis* infects approximately 7 million people in China and other parts

of the Far East. Liver flukes usually enter human's gastrointestinal tract after ingestion of raw fish, and the parasites then travel via the duodenum into the host's intrahepatic or extrahepatic biliary ducts. Liver flukes cause bile stasis, inflammation, periductal fibrosis, and hyperplasia, with the subsequent development of cholangiocarcinoma.

Perspectives

Infectious agents play a major role in human cancer, either as direct or indirect carcinogens. This chapter reviews the association of a number of agents that have been generally accepted as playing a major role in human cancer. The controversy surrounding SV40 as a potential oncogenic agent is also discussed. Other agents have been implicated in the literature; however, data for these agents are not compelling. Different infectious agents contribute to carcinogenesis in different ways. Some like HPV and cervical cancer and HTLV-1 and ATL do so in a direct manner through oncogenic proteins encoded by the virus. Others like the liver flukes and cholangiocarcinomas and *S. haematobium* and bladder cancer likely do so through indirect mechanisms involving persistent infection and inflammation. The criteria that are generally used to determine whether an agent is carcinogenic must involve a combination of epidemiology and molecular biology. Several questions arise. Are there additional unknown infectious agents associated with human cancer? If so, what cancers and how can they be discovered? Certainly cancers in immunologically compromised individuals are good candidates for an infectious etiology. Advances in array technologies and bioinformatics searching tools should provide important platforms to examine such cancers. Another question is whether some ubiquitous infectious agents might contribute to the initiation of some cancers, but do so in a hit-and-run fashion such that a molecular fingerprint is not left behind. How will the role of such agents in human cancers be discovered?

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Environmental Carcinogenesis

Introduction to Cancer and the Environment

Environment, Genetics, and Cancer

Overall human cancer risk is determined by complex interactions between host genetics and environmental exposures. Upon exposure to a cancer-causing agent, a cascade of events is set into motion that converts normal cells into cancer cells. This process is referred to as carcinogenesis, and cancer-causing agents are referred to as carcinogens. Hundreds of confirmed and suspected environmental carcinogens have been identified. Exposure to a variety of natural and synthetic substances in the environment is believed to account for up to two-thirds of cancer mortality worldwide. In the context of the current chapter we refer to the “environment” as any substance or agent that is normally present outside of the human body and that interacts with the human body to increase cancer risk. Genetically controlled host factors also contribute to cancer risk primarily through modulation of responses to environmental agents. Understanding the causes of cancer and the underlying mechanisms that lead to cancer development provides a rational basis for developing prevention strategies. In this chapter, we discuss the major known environmental causes of cancer and, where applicable, underlying mechanisms. In addition, where known, significant gene–environment interactions are highlighted.

History of Chemicals and Cancer

Environmental contribution to disease, cancer in particular, has been recognized for centuries (1). In 1775, Dr. Percival Pott made the observation that chimney sweeps had an increased incidence of scrotal cancer likely caused by exposure to soot. A century later, skin cancers related to occupational exposure were reported in coal tar workers in Germany. In 1915, these observations were experimentally validated by Yamagiwa and Ichikawa, who demonstrated that multiple topical applications of coal tar to rabbit ears induced skin carcinomas. These were the first studies to demonstrate chemical induction of cancers. These findings were further refined in a series of studies conducted by Sir Ernest Kennaway and others in the 1920s and 1930s. The studies demonstrated that the carcinogenic activity of coal tar resided in a compound consisting

entirely of carbon and hydrogen and demonstrating a characteristic fluorescent spectrum. Ultimately, benzo[*a*]pyrene (B[*a*]P) (Figure 7-1) was identified as the major active carcinogen in coal tar possessing the characteristic fluorescent spectrum. B[*a*]P is one of a number of carcinogenic polycyclic aromatic hydrocarbons (PAHs) formed by incomplete combustion of organic molecules. In isolating a compound from coal tar that could induce cancer in animals, an occupational carcinogen exposure was linked to cancer incidence, and the utility of animal models of carcinogenicity in the interpretation of human epidemiologic trends was established.

Causes of Cancer

Epidemiology and Causal Criteria

Due to correlative epidemiologic data analysis, cancer risk is known to vary extensively worldwide. For instance, liver cancer incidence is highest in eastern Asia and lowest in Northern Europe and Central America. Prostate cancer rates are high in the United States, Canada, and Scandinavia, especially in comparison with the rates in China and other Asian countries. Similarly, breast cancer risk is higher in the United States and European countries than in China and India. Capitalizing on known ethnic variation in cancer rates, analysis of cancer risk in migrant populations has been undertaken and has yielded important information concerning the relative contribution of environment versus genetics in cancer etiology. In these studies, the rate of cancer in migrant cohorts is compared with the rate of cancer among people of the same ethnicity living in the country of origin and to the cancer rate of people in the destination population. For example, breast cancer incidence among Asian immigrants to the United States has been compared with that of women still living in their country or region of origin (2). The breast cancer risk of Asian-American women born in the East has been shown to rise with increasing number of years lived in the West. Ultimately, the risk of breast cancer among Asian-American women approaches that of U.S.-born caucasian women and is significantly higher than Asian women still living in the country of origin. Numerous studies of this kind demonstrate that even while in the first generation following relocation, immigrant populations demonstrate a pattern of cancer risk in common with native populations rather than with populations in their country of origin.

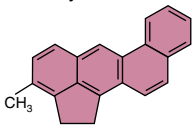
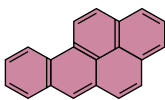
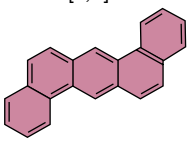
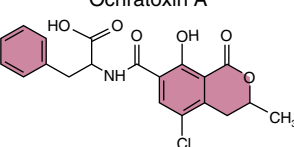
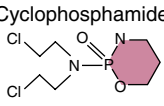
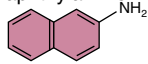
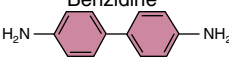
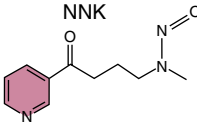
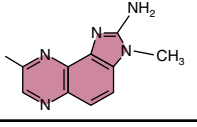
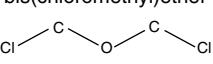
CARCINOGEN CLASS	REPRESENTATIVE EXAMPLE
PAHs	3-methylcholanthrene 
	Benzo[a]pyrene 
	Dibenzo[a,h]anthracene 
Solvents	Benzene 
Mycotoxins	Ochratoxin A 
Alkyl halides	Vinyl chloride 
	Cyclophosphamide 
Aromatic amines	2-naphthylamine 
	Benzidine 
N-nitrosamines	NNK 
Heterocyclic amines	MeIQx 
Alkylating agents	bis(chloromethyl)ether 

FIGURE 7-1 Chemical structures of selected carcinogens.

These studies imply that environmental factors play a major role in determining cancer risk. Similarly, studies of cancer risk in twins have suggested that gene inheritance plays a lesser role in determining cancer development than do environmental factors.

As a consequence of unfortunate exposure incidents, human epidemiologic studies have identified numerous environmental causes of cancer. In combination with available mechanistic data, epidemiologic data can be analyzed for likelihood that cancer risk is related to a particular environmental exposure. The strength of evidence for a causal role in cancer development can be evaluated using criteria developed as a modification of Bradford-Hill's criteria (1965) for assessment of evidence of causation (3):

1. Strength of Association: Large-magnitude effects on cancer risk are less likely than small magnitude effects to be due to chance.
2. Temporal Relationship: To be causal, the environmental exposure must have happened in advance of appearance of cancer.
3. Biologic Plausibility: Relationships that can be supported by laboratory evidence or a plausible hypothesis are more likely to be causal relationships.
4. Dose-Response Relationship: Studies that demonstrate a gradient in disease outcome whenever a gradient in exposure has occurred provide stronger support for a causal relationship than those studies that do not demonstrate a dose-response relationship.
5. Consistency: The strongest causal relationships are consistently demonstrated in multiple studies of the exposure-disease relationship.

Using these criteria, numerous cancer-causing agents and/or risk factors have been identified for further characterization.

Known Cancer Risk Factors

Epidemiologic studies of cancer deaths, such as those used in the landmark paper published by Doll and Peto in 1981 (4), have attempted to identify causative agents and lifestyle choices that determine cancer risk. Doll and Peto identified two environmental factors to which as many as 60% of all cancer deaths can be attributed: diet and tobacco use. More than 20 years later, most of Doll and Peto's estimates have only been strengthened by additional evidence. Tobacco use is believed to contribute to at least 30% of all cancer deaths. Dietary components and factors are also believed to contribute greatly to overall cancer death rates. Additional factors cited by multiple regulatory agencies as contributing to cancer risk include occupation, geophysical factors, alcohol, pollution, infections, medications, and reproductive factors.

Smoking

Tobacco use remains as the single most important and avoidable factor in determining cancer risk (5). Lung, bladder, esophageal, pancreatic, liver, oral, and nasal cavity cancers, among others, have all been associated with tobacco use. It has been estimated that

90% of all lung cancer deaths in males can be attributed to smoking. Lung cancer risk is greatest for persons who begin smoking at an early age and continue smoking for many years, and the risk of tobacco smoke-induced lung cancer is directly proportional to the dose inhaled. Tobacco smoke is a complex mixture of chemicals, 55 of which are known or suspected human carcinogens (Table 7-1). Upon absorption in the lungs, these agents may act locally or at distal sites to (1) induce DNA damage and (2) alter cellular growth and proliferation. A synergistic effect has been noted in the case of combined tobacco use and heavy alcohol use. Despite antitobacco sentiment, one fourth of U.S. citizens are still smokers, and smoking rates in countries such as China remain high; therefore, smoking-induced cancers are likely to continue to be prevalent worldwide.

Diet

Second only to tobacco usage, diet is a critical determinant of cancer risk. This risk has been attributed both to dietary chemical constituents and to overall energy consumption. As much as 14% to 20% of cancer deaths have been attributed to overweight and obesity. Overweight and obesity, as defined by the ratio of weight to height known as body mass index (BMI), are on the rise in the United States and other developed countries. Traditionally, overweight and obesity have been associated with elevated risk of cancers of the colon, breast, endometrium, kidney, and esophagus. A prospective cohort study of cancer mortality and BMI in a study population of over 900,000 U.S. adults confirmed these findings and identified non-Hodgkin lymphoma, multiple myeloma, liver, pancreas, gallbladder, stomach, and ovarian cancers as obesity-related cancers (6). Additionally, animal studies have consistently

demonstrated that restricting calorie intake can significantly reduce cancer risk, while inducing obesity can significantly elevate cancer risk. Despite these suggestive findings, the exact mechanistic basis for the effect of calorie intake on cancer formation is unknown (7). Elevated steroid hormone production in adipose tissue has been proposed as the basis for obesity-induced endometrial and breast cancers. Additionally, dysregulation of insulin and IGF-1 levels in obese individuals may contribute to cancer development. In general, cancers due to obesity can be expected to rise in coming years since the obesity rate in many regions of the United States and in other countries has risen dramatically in recent years.

In addition to excess calorie intake, certain dietary constituents may affect cancer risk (8). For example, red meat consumption has been associated with elevated colorectal cancer risk possibly due in part to the carcinogenic nitrosamine and heterocyclic amine content of preserved or heat-treated meats. Fungal toxins such as aflatoxins are food contaminants resulting from mold growth on foodstuffs. Several of these toxins have been shown to be extremely potent mutagens and in some cases potent carcinogens (e.g., aflatoxin B₁ [AFB₁]). On the contrary, in the United States, cancer risk due to food additives is presumed to be quite low since the U.S. Food and Drug Administration (FDA) strictly regulates food additive use. In 1958, an amendment to the Food, Drugs, and Cosmetic Act of 1958, referred to as the Delaney Clause, was approved and stated that “the Secretary (of the FDA) shall not approve for use in food any chemical additive found to induce cancer in man, or, after tests, found to induce cancer in animals.” Presumably, therefore, cancer risk due to food additive consumption is quite low. Also, although examples of carcinogenic dietary constituents can be identified, a possibly greater dietary determinant of cancer risk is consumption of anticarcinogenic fruits and vegetables. Consumption of fruits and vegetables has consistently been linked to reduced cancer risk for a variety of cancer types.

Table 7-1 Carcinogens in Tobacco Smoke^a

Carcinogen Class	No. of Compounds	Example Compound
Polycyclic aromatic hydrocarbons	10	B[a]P 5-Methylchrysene Dibenz[a,h]anthracene
Aza-arenes	3	Dibenz[a,h]acridine
N-nitrosamines	7	NNK N-Nitrosodiethylamine
Aromatic amines	3	4-Aminobiphenyl
Heterocyclic amines	8	2-amino-3-methylimidazo [4,5-f]quinoline
Aldehydes	2	Formaldehyde
Miscellaneous organic compounds	15	1,3-Butadiene Ethyl carbamate
Inorganic compounds	7	Nickel Chromium Cadmium Arsenic
Total	55	

^a Adapted from Hecht SS. Tobacco smoke carcinogens and lung cancer. *J Natl Cancer Inst* 1999;91:1194.

Occupation

Many carcinogens have been identified at the cost of human exposure and cancer incidence that occurred as a result of industrialization. Human epidemiologic studies highlight the potency of chemical and physical carcinogens and how lack of understanding leads to lack of preparation and protection (9–11). In the 1800s, high incidence of bladder cancer among workers in the aniline dye industry was recognized. Later, evidence demonstrating that 2-naphthylamine and benzidine were two carcinogenic agents responsible for the cancer incidence was reported. Also during the early 1900s, nearly 5,000 workers were hired to apply luminous radium-containing paint to watch and instrument dials. Due to their occupational radiation exposure and a lack of precautionary practices, a large excess of bone cancers was noted among this cohort. Thousands of workers were exposed to vinyl chloride before its ability to induce angiosarcoma of the liver was recognized. Since the 1970s, strict workplace regulations and protective measures in the United States have largely prevented such happenings. The Occupational Safety and Health Administration (OSHA) was signed into existence in 1970 by the U.S. government with the goal of ensuring worker safety and

Table 7-2 Environmental Carcinogens Identified Via Associations with Occupation

Occupation	Carcinogen Exposure	Associated Cancer Type
Iron and steel founding	PAH, chromium, nickel, formaldehyde	Lung
Copper mining and smelting	Arsenic	Skin, bronchus, liver
Aluminum production	PAH	Lung
Coke production	PAH	Lung, kidney
Painting	Chromium, solvents	Lung
Furniture and cabinet making	Wood dust	Nasal sinus
Boot and shoe manufacture	Leather dust, benzene	Nasal sinus, leukemia
Rubber industry	Aromatic amines, solvents	Bladder, leukemia,
Nickel refining	Nickel	Nasal sinus, bronchus
Vinyl chloride manufacture	Vinyl chloride	Liver
Dye and textile production	Benzidine-based dyes	Bladder

PAH, polycyclic aromatic hydrocarbons.

health by improving workplace environment. OSHA sets the legal limit for worker exposure to hazardous compounds in the United States. These limits are referred to as permissible exposure limits (PEL). PELs have been issued for approximately 500 chemicals, a portion of which are known or suspected carcinogens. Also created in 1970, the Environmental Protection Agency (EPA) is charged with protecting human health and the environment. In addition to other roles, the EPA regulates the release of industrial pollution, including carcinogens. Before these institutions were in place, employment in a wide variety of settings was linked to elevated risk of numerous cancers (Table 7-2).

Table 7-3 IARC Classification of Suspected Carcinogenic Agents

Group 1: Carcinogenic to humans: Sufficient evidence of carcinogenicity in humans exists or sufficient evidence of carcinogenicity in animals is supported by strong evidence of a relevant mechanism of carcinogenicity in humans.

Group 2A: Probably carcinogenic to humans: Limited evidence of carcinogenicity in humans exists but sufficient evidence of carcinogenicity in animals has been demonstrated. Alternatively, inadequate evidence in humans with sufficient evidence in animals may be supported by strong evidence that a similar mechanism of carcinogenicity would occur in humans.

Group 2B: Possibly carcinogenic to humans: Limited evidence of carcinogenicity in humans exists but inadequate evidence in experimental animals. Alternatively, this classification can be used for agents for which there are inadequate data in humans but sufficient evidence in animals or strong mechanistic data.

Group 3: Unclassifiable as to carcinogenicity in humans: Inadequate evidence in humans and animals exists. Alternatively, sufficient evidence of carcinogenicity may exist in animals but strong mechanistic data predict a lack of carcinogenicity in humans.

Group 4: Probably not carcinogenic to humans: Evidence suggesting a lack of carcinogenicity in humans and animals exists.

IARC, International Agency for Research on Cancer.

Classes and Types of Carcinogens

Carcinogen Evaluation and Classification

The National Toxicology Program (NTP), in cooperation with various governmental agencies such as the EPA and the World Health Organization (WHO) International Agency for Research on Cancer (IARC), both produce reports listing known and suspected human carcinogens. These documents provide critical information summarizing evidence of carcinogenicity for all known carcinogens. This information is used both to educate the public and guide exposure limit regulation. IARC *Monographs on the Evaluation of Carcinogenic Risks to Humans* identify carcinogens, defined as agents “capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity.” Agents are selected for evaluation on the basis of two factors: (1) evidence of potential carcinogenicity and (2) known exposure of humans. During the scientific review and evaluation of potential carcinogens, a working group is formed and charged with summarizing available data concerning anticipated exposure levels, human epidemiologic data, and studies of cancer-producing capacity in animals. Although the goal of IARC *Monographs* has been to identify carcinogens regardless of an explanatory mechanism, information on mechanisms can also be used as supporting data. All agents evaluated by IARC are classified into one of five categories as shown in Table 7-3. As of the most recent report, 100 agents, groups of agents or exposure scenarios are listed as “Carcinogenic to Humans” (a partial listing is shown in Table 7-4). An additional 68 are listed as “Probably Carcinogenic to Humans.” These agents are extremely diverse in structure, potency, and mechanism.

Types of Carcinogens

Carcinogens can be grouped into one of three categories: (1) physical carcinogens, (2) biologic carcinogens, and (3) chemical carcinogens. The term “physical carcinogen” encompasses multiple types of radiation (e.g., ultraviolet [UV] and ionizing radiation). Biologic carcinogens refer to viral and bacterial infections that have been associated with cancer development (e.g., human papilloma virus [HPV] and hepatitis B virus [HBV]). Most carcinogens can be categorized as chemical carcinogens. In addition to heavy metals, organic

Table 7-4 Selected IARC Known Human Carcinogens

4-Aminobiphenyl	Hepatitis B virus
Arsenic	Hepatitis C virus
Asbestos	Human immunodeficiency virus type 1
Azathioprine	Human papillomavirus
Benzene	Human T-cell lymphotropic virus
Benzidine	Melphalan
Benzo[a]pyrene	8-Methoxypsoralen
Beryllium	Mustard gas
<i>N,N</i> -Bis(2-chloroethyl)-2-naphthylamine	2-Naphthylamine
Bis(chloromethyl)ether	Nickel compounds
Chloromethyl methyl ether	<i>N</i> '-Nitrosomonicotine (NNN)
1,4-Butanediol dimethanesulfonate	Phosphorus-32
Cadmium	Plutonium-239
Chlorambucil	Radioiodines
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea	Radium-224
Chromium[VI]	Radium-226
Ciclosporin	Radium-228
Cyclophosphamide	Radon-222
Diethylstilboestrol	Silica
Epstein-Barr virus	Solar radiation
Erionite	Talc containing asbestiform fibres
Estrogen-progestogen menopausal therapy	Tamoxifen
Estrogen-progestogen oral contraceptives	2,3,7,8-Tetrachlorodibenzo- <i>para</i> -dioxin
Estrogen therapy	Thiotepa
Ethylene oxide	Treosulfan
Etoposide	Vinyl chloride
Formaldehyde	X- and Gamma (g)-Radiation
Gallium arsenide	Aflatoxins
<i>Helicobacter pylori</i>	Soots
	Tobacco
	Wood dust

IARC, International Agency for Research on Cancer.

combustion products (e.g., B[a]P), hormones, and fibers (e.g., asbestos), among others, are considered to be chemical carcinogens. Note that in the discussion that follows, only selected carcinogens that are known to be carcinogenic in humans are described (Table 7-4). For a more comprehensive listing of carcinogenic agents, including those listed in other IARC categories refer to the WHO IARC monograph database (<http://monographs.iarc.fr/ENG/Monographs/allmonos90.php>) and additional references (12,13).

Physical Carcinogens

Examples of physical carcinogens include UV and ionizing radiation. Radiation refers to flow of energy-bearing particles; ionizing radiation refers to radiation that is of sufficiently high energy to remove an electron from an atom or molecule with which it collides. Exposure to ionizing radiation of various forms has been shown to cause multiple forms of cancer. Additionally, solar radiation, in particular UV radiation, has sufficient energy to cause photochemical damage, leading to skin cancer formation.

The incidence of skin cancers such as melanoma, basal cell carcinoma and squamous cell carcinoma has risen dramatically in recent years (14). The risk of developing skin cancer is highest in equatorial regions and correlates with the number of blistering sunburns encountered during childhood. Correlative studies such as these, in addition to mechanistic studies at the cellular and organismal levels, indicate that most skin cancers arise due to exposure to solar radiation. In particular, UV radiation, which spans the 100- to 400-nm range, appears to be causative (15). The health effects

of UV radiation vary according to wavelength. Consequently, UV radiation is divided into three regions: UVA, 315 to 400 nm; UVB, 280 to 315 nm; UVC, 100 to 280 nm. UVB and UVA are relevant to cutaneous carcinogenesis since, as opposed to UVC, radiation at these wavelengths can bypass the earth's atmosphere, including stratospheric ozone. UVB is differentiated from UVA in that moderate UVB exposure results in an erythematic response, and UVB is well absorbed by cellular molecules such as DNA, melanin, amino acids, carotene, and urocanic acids (16,17). UVB is most potent in inducing skin tumors in hairless mice. However, exposure to all UV wavelength ranges results in DNA damage and mutation in *in vitro* models, and UVA also induces tumors in hairless mice.

For UV radiation to produce a skin response, photon energy must be absorbed into the chemical bonding of these target biomolecules; melanin is a critical UV radiation absorption filter while DNA is a major target for carcinogenic effects. UV irradiation of DNA results in formation of pyrimidine dimers in addition to other photodamage such as DNA strand breaks and pyrimidine-pyrimidone photoproducts. When these lesions are not repaired, DNA mutations can result. The hallmark UV radiation-induced lesions are C→T or CC→TT transitions. Target genes for solar radiation-induced mutations include *p53* (squamous cell carcinomas [SCCs] and basal cell carcinomas [BCCs]), *p16* (melanoma), and *PTCH* (BCCs, possibly SCCs). UV irradiation of skin keratinocytes also alters numerous cell signaling pathways such as growth arrest and DNA damage-response genes (i.e., *p53*, *GADD45*, mismatch repair genes), apoptosis signaling molecules (i.e., *bcl-2*, *fas*), and mitogenic signals (i.e., *ras*, *ERK*).

In addition to solar radiation, ionizing radiation in the form of x-rays, nuclear fallout, and therapeutic irradiation as well as energy deposition from radon gas also contribute to incidence of human cancers. Epidemiologic studies of radiation workers and atom bomb survivors of Hiroshima and Nagasaki as well as the use of animal models have led to the characterization of ionizing radiation as a “universal carcinogen” (18). Ionizing radiation can induce tumors in most tissues and in most species examined due to its unique ability to penetrate tissues and induce DNA damage via energy deposition (19).

Radon-222 is a radioactive gas that is produced by radioactive decay of uranium-238 and is found ubiquitously in soil and rock. Concern over accumulation of radon in indoor air, especially in underground spaces, has led to study of the health effects of inhaled radon. Radon decay results in the release of α particles, two protons and two neutrons, which do not deeply penetrate tissues but possess the capacity to damage DNA in areas of contact. Inhalation of radon results in decay product exposure of the bronchial epithelium and has been associated with lung cancer incidence; however, the carcinogenic potential of α particle radiation remains controversial, especially at the low exposure level of radon in homes (20).

Biologic Carcinogens

Biologic carcinogens also play an important role in human carcinogenesis. Approximately 20% of human cancers are associated with infectious agents including bacteria, parasites, and viruses. These are discussed in more detail in Chapter 6.

Chemical Carcinogens

Chemical carcinogens can be classified according to their chemical nature: organic, inorganic, fibers, and hormones. The first experimental confirmation of the existence of organic chemical carcinogens began in 1915 when Yamagiwa and Ichikawa demonstrated that multiple applications of coal tar could induce skin tumors in rabbits (1). It was later shown that the active carcinogenic agent was composed entirely of carbon and hydrogen. Since that time numerous carbon-based carcinogens have been identified in studies of experimental animals and of human epidemiologic data. These chemicals range from industrially produced and utilized solvents to naturally occurring but chemically complex combustion products and mycotoxins to simple alkyl halides such as vinyl chloride (Figure 7-1).

Organic Carcinogens

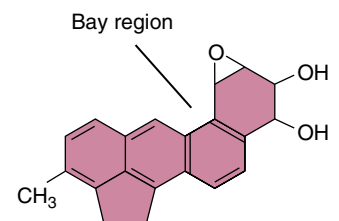
Benzene

Benzene is a widely used solvent and is present in gasoline, automobile emissions, and cigarette smoke. Historically, high-level exposure to benzene was commonplace, and, in general, benzene exposure has been the cause of great concern due to its carcinogenic properties. Exposure to benzene occurs in industrial settings such as in rubber production, chemical plants, oil refineries, and shoe manufacturing. Since benzene is a volatile aromatic solvent, inhalation exposures predominate (21).

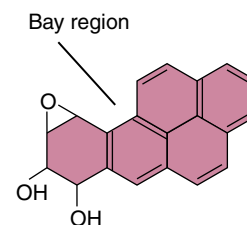
The carcinogenic properties of benzene have long been recognized; an increased risk of leukemia has been shown in workers exposed to high levels of benzene. The strongest associations of benzene and cancer risk are found with risk of acute myeloid leukemia and to a lesser extent, chronic lymphocytic leukemia. The precise mechanisms whereby benzene induces leukemia are unknown; however, benzene is a recognized clastogen (22). Workplace exposure restrictions have reduced human exposure to high levels of benzene. Current research is aimed at assessing risk associated with chronic low-level exposure scenarios.

Polycyclic Aromatic Hydrocarbons

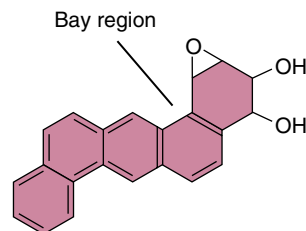
Polycyclic aromatic hydrocarbons (PAHs) refer to a diverse group of intensively studied organic compounds. Many PAHs can be metabolically activated to become highly reactive, electrophilic mutagens. PAHs are converted to “bay region” diol epoxides as depicted in Figure 7-2. These diol epoxides are able to form covalent adducts with DNA, and their overall reactivity is related to carcinogenic potency (23). For example, benzo[*a*]pyrene diol-epoxide reacts extensively with the exocyclic amino group of guanine to produce mutagenic DNA adducts (Figure 7-3 and see section entitled Initiation and Mutational Theory of Carcinogenesis). Additionally, certain PAH metabolites may act synergistically



3-methylcholanthrene diol epoxide



Benzo[*a*]pyrene diol epoxide



Dibenz[*a,h*]anthracene diol epoxide

FIGURE 7-2 Selected polycyclic aromatic hydrocarbons (PAH) bay region dihydrodiol epoxides.

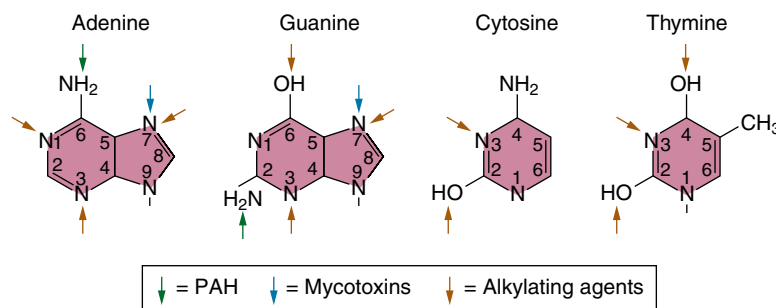


FIGURE 7-3 Sites of adduct formation associated with carcinogenesis of selected agents.

with bay region diol epoxide metabolites to promote tumor formation in a manner unrelated to DNA adduct formation (24). PAHs are formed during combustion of organic matter such as coal, mineral oil, and oil shale. Therefore, PAH exposure occurs in the form of automobile exhaust, soot, coal tar, and cigarette smoke and in charred food products. Many PAHs have been found to be carcinogenic in animal studies, and PAH exposure is associated in humans with lung, skin, and urinary cancers among others. The carcinogenic potential of PAHs is highly variable. Examples of potent to moderately carcinogenic PAHs include 3-methylcholanthrene, B[a]P, dibenzo[a,b]anthracene, 5-methylchrysene, and dibenz[a,j]anthracene whereas benzo[e]pyrene, dibenz[a,c]anthracene, chrysene, benzo[c]phenanthrene and fluorene are relatively weak or inactive carcinogens. Furthermore, humans are exposed to mixtures of PAH that are produced during combustion of organic material.

Aflatoxin B₁

One of the most potent liver carcinogens is the fungal metabolite, aflatoxin B₁ (AFB₁). AFB₁, as well as related aflatoxin compounds, are produced by *Aspergillus* mold species, such as *Aspergillus flavus* and *Aspergillus parasiticus*. Exposure to aflatoxins occurs via consumption of contaminated nuts and grain, such as corn and peanuts, on which *Aspergillus* species grow. Humid conditions and poor storage contribute to growth of these molds. In numerous epidemiologic studies, the incidence of hepatocellular carcinoma (HCC) has been correlated with aflatoxin intake. AFB₁ is highly mutagenic in *in vitro* assays. AFB₁ is converted to an epoxide metabolite responsible for its carcinogenic action (25). The base targeted by activated AFB₁ is G (N7 position (Figure 7-3)), and the mutations induced are predominantly GC→TA transversions (26). Significantly, the *p53* gene is mutated (GC→TA point mutation in codon 249) in a high proportion of human HCCs that arise in areas where aflatoxin exposure is high. Evidence suggests that *p53* mutation at codon 249 may occur as a result of combined exposure to HBV and AFB₁, and studies have shown elevated risk of HCC in individuals exposed to both HBV and aflatoxin over individuals exposed to either agent alone (27).

Benzidine

Benzidine is a member of a large class of carcinogens referred to as aromatic amines. The carcinogenic nature of benzidine was

discovered in the context of bladder cancer induction in workers in the dye industry (28). In the past, benzidine-based azo dyes were synthesized in vast quantities in the United States and abroad. In the 1970s, their use was significantly curtailed due to health concerns. However, numerous workers were exposed to these carcinogens before regulation. Upon activation, benzidine and certain benzidine-based dyes can covalently react with DNA, and benzidine has been shown to induce chromosomal damage *in vivo* (29). Benzidine has also been shown to be a bladder carcinogen in multiple species including humans, dogs, mice, rats, and hamsters, although species differences in activation of the parent compound have made the study of benzidine-induced bladder cancer challenging (30).

Nitrosamines and Heterocyclic Amines

N-nitroso compounds such as *N*-nitrosamines are powerful carcinogens in multiple species and are suspected gastrointestinal carcinogens in humans (31). Following metabolic activation *N*-nitrosamines can react with DNA to initiate carcinogenesis. Exogenous and endogenous sources of *N*-nitroso compounds have been described. *N*-nitrosamines are present in smoked meats and in meats containing the antimicrobial and color-enhancing agent, nitrite. In both cases, nitrogen oxides are formed, which react with the amines present in meat. Alternatively, formation of *N*-nitroso compounds can occur endogenously due to low pH conditions in the gastric system or due to the presence of intestinal bacteria that catalyze *N*-nitroso compound formation.

Heterocyclic amines are also formed in muscle meats upon high-temperature processing. Most heterocyclic amines tested are mutagenic in *in vitro* assays, and several induce gastrointestinal tumors in rodents (32). The two heterocyclic amines found most abundantly in cooked meat and best absorbed into circulation are 2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine (PhIP) and 2-amino-3,8-dimethylimidazo (4,5-f) quinoxaline (MeIQx). At high temperatures, heterocyclic amines are formed via reactions among creatinine, creatine, sugars, and amino acids.

N-nitrosamine exposure is also associated with tobacco use (33): 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and *N*-nitrosornicotine (NNN) are carcinogenic tobacco-alkaloid-derived *N*-nitrosamines present in unburned and burned tobacco

products. PAHs and NNK are the most abundant pulmonary carcinogens in tobacco smoke. As opposed to PAHs, which induce SCCs, NNK induces adenocarcinoma of the lung in animal models. As opposed to SCC, adenocarcinoma of the lung has become the most common lung cancer type in the United States. This fact may reflect changes in cigarette manufacturing in the last 30 to 40 years that have resulted in rising levels of NNK and falling levels of B[a]P. Additionally, in smokeless tobacco products such as snuff, *N*-nitrosamines are prominent agents involved in the induction of oral cancer. These *N*-nitrosamines require metabolic activation for carcinogenic activity and DNA adduct formation similar to other organic carcinogens discussed above.

Inorganic Carcinogens

Beryllium

In 1946, Hardy and Tabershaw reported “delayed chemical pneumonitis” in workers exposed to beryllium (reviewed in [34]). In that same year, Gardner and Heslington reported experimentally induced osteosarcomas in beryllium-injected rabbits. Subsequent studies in the 1950s demonstrated that inhalation exposure of rodents resulted in induction of lung tumors. Since that time, beryllium has been recognized as a human carcinogen capable of inducing lung cancer in exposed workers. Occupational exposures to beryllium include inhalation of beryllium-containing dusts during processing of ores, machining of beryllium metal and alloys, and manufacturing of aerospace materials, ceramics, sports equipment, and electronics. Beryllium is weakly mutagenic in bacterial and mammalian mutagenesis test systems; however, it shows strong transformation capacity in Balb/3T3 and Syrian hamster secondary embryo cells [35]. In addition to genotoxic effects, beryllium has been shown to alter expression of numerous cancer-related genes (i.e., *c-fos*, *c-jun*, *c-ras*), DNA repair genes, and genes within the MAP kinase pathway.

Cadmium

Cadmium is a heavy metal present in soil, air, and water and is listed as a priority pollutant by the U.S. EPA. Occupational exposures to cadmium occur during the manufacture of nickel-cadmium batteries, pigments, and plastic stabilizers as well as electroplating processes, metal smelting, and electronic waste recycling [36]. Additionally, cigarette smoke contains cadmium. Release of industrial cadmium waste into the environment is of particular concern due to the long half-life of cadmium. Similarly, cadmium can accumulate in the body since it is poorly excreted and effectively stored due to binding to metallothionein. The half-life of cadmium in humans is estimated at 15 to 20 years. Furthermore, once absorbed, no effective detoxification pathways for cadmium exist.

Cadmium exposure has been linked to human lung cancer and may affect risk of prostate, pancreas, and kidney cancers. Although the carcinogenicity of cadmium has been confirmed in rodent models, the precise mechanism is unknown [37]. Cadmium binds only weakly to DNA, is only weakly mutagenic in bacterial and mammalian assays, and high concentrations are required to induce oxidative stress. Cadmium may work via epigenetic mechanisms to activate proto-oncogenes and disrupt normal cellular processes. For example, cadmium has been shown to alter

E-cadherin-mediated cell adhesion, inhibit DNA repair, and alter expression of numerous genes *in vitro* including *c-fos*, *c-myc*, metallothionein, and genes encoding heat shock proteins [36].

Arsenic

Arsenic is widely distributed in the environment, being found in the earth's crust in both inorganic [arsenite-As(III) and arsenate-As(V)] and methylated forms (monomethylated arsenic [MMA] and dimethylated arsenic [DMA]). As(III), as well as MMA(III) and DMA(III), have been associated with skin, lung, urinary bladder, kidney, and liver cancers [38]. Human exposure to arsenic occurs via contaminated drinking water, diet, contact with wood preserved with arsenicals; during mining of tin, gold, and uranium; and during application of arsenical pesticides. Signs of chronic exposure to arsenic in drinking water include altered skin pigmentation and hyperkeratosis of the palms of the hand and soles of the feet, which may ultimately lead to skin lesions and skin cancer.

Much attention has been given to assessing the health impact of arsenic contamination in drinking water sources. The current WHO guidelines for arsenic exposure recommend no more than 10 $\mu\text{g/L}$ arsenic in water intended for human consumption. Since the 1980s, millions of people in China, India, Bangladesh, the United States, Chile, and Argentina have been exposed to arsenic in the drinking water far in excess of this limit. Already, numerous epidemiologic studies in Taiwan, the United States, Chile, and Argentina have demonstrated excess cancer risk in areas with known high exposure to arsenic in drinking water [39]. Unfortunately, identifying a safe level of arsenic in drinking water has been difficult because most epidemiologic studies show adverse effects at high doses; data concerning health risk at low exposures are unavailable. After intense debate, the limit in the United States was lowered to 10 $\mu\text{g/L}$ in 2001.

As(III) and As(V) are transported into cells, As(III) more readily than As(V). Upon absorption, As(V) is reduced to As(III); As(III) can then be methylated. Historically, methylation of As(III) was considered to be a detoxification reaction but recent evidence contradicts this dogma [40]. MMA(III) and DMA(III) are more cytotoxic, mutagenic, and clastogenic than As(III). However, when methylated, arsenic is readily excreted in urine. Therefore, several useful biomarkers of arsenic exposure are available. DMA can be detected in urine shortly after exposure; additionally, due to the wide distribution of arsenic, exposure can be assessed via hair and finger nail deposits months or years after exposure.

Numerous mechanisms of action have been proposed for arsenic carcinogenicity [38]. Arsenic exposure is known to generate reactive oxygen species. Like many transition metals, arsenic can participate in Fenton reactions that produce oxidative stress. Furthermore, arsenic may activate superoxide-generating NAD(P)H oxidase. In this way, arsenic is thought to induce DNA and protein damage that may initiate carcinogenesis. Arsenic has also been shown to elevate the total level of tyrosine phosphorylation in cells. Specifically, arsenic may alter phosphorylation-dependent epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) signaling. Additionally, arsenic has been shown to alter NF- κ B signaling, apoptosis rates, cell cycle regulation, DNA methylation, and genome stability.

Chromium

Chromium in the hexavalent state (Cr(VI)) is a human carcinogen. The carcinogenic properties of chromium have been identified via epidemiologic studies of exposed workers in industries such as chrome plating, welding, leather tanning, and stainless steel production (41). Exposure to chromium generally occurs via inhalation and primarily affects risk of lung cancer. Due to environmental contamination, consumption of chromium in drinking water is also possible; however, the health consequences of the low-level exposure are unclear.

The oxidation state of chromium determines not only its bioavailability but also its cellular reactivity (42). Cr(VI) readily enters cells via anion channels whereas Cr(III) only slowly crosses the cell membrane. Upon entry to the cell, Cr(VI) is likely reduced, since Cr(VI) does not readily react with DNA in *in vitro* analyses. Chromium in lower oxidation states [Cr(III), Cr(IV) and Cr(V)] is more reactive; Cr(III) is believed to be the ultimate DNA reactive form (41). The reduced forms of chromium can also induce oxidative stress. In addition to or as a result of oxidative stress, chromium alters cell signaling pathways. Signaling molecules affected include NF- κ B, AP-1, p53 and HIF-1.

Fibers

Asbestos

The term “asbestos” refers to a group of naturally occurring silicate mineral fibers. There are numerous types of asbestos fibers that are classified according to their morphologic characteristics, including whether the fibers are curly (serpentine) or straight (amphibole). The shape and length-to-width ratio are important determinants of whether a particular asbestos fiber type will be carcinogenic (43). This is likely because the size of the fiber determines the ability of the fiber to reach the deep lung tissues and penetrate the lung. Long ($>4\ \mu\text{m}$) and thin ($<0.5\ \mu\text{m}$ diameter) fibers are the most carcinogenic. Extensive exposure to asbestos has occurred because the flame-resistant and durable characteristics of asbestos have led to its use as an insulating agent in schools, factories, homes, and ships, as construction material, and as a raw material for automobile brake and clutch parts. A large cohort of workers was exposed to high levels of asbestos when ship-building peaked during World War II.

The toxic effects of asbestos exposure have been known for many years (43). For example, more than 40 years ago, crocidolite asbestos exposure of South African miners was linked to mesothelioma incidence. Mesothelioma is a rare cancer of membranous lining of the abdomen and chest. Numerous animal studies and *in vitro* experiments support the conclusion that asbestos can induce tumors. In fact, few cancer cause–effect relationships are as striking as asbestos and mesothelioma; most cases of mesothelioma can be related to asbestos exposure. In addition to mesothelioma, asbestos exposure has been associated with lung and larynx cancers. Since identification of asbestos as a cancer-causing agent, asbestos usage in the United States has greatly declined due to the introduction of a replacement material (fiberglass) and OSHA regulation of asbestos exposure. However, the initial accumulation of evidence of asbestos carcinogenicity was obscured by the differences in carcinogenicity of asbestos

fibers of varying shape and by the long latency for development of tumors following exposure.

Numerous biologic hypotheses concerning the mechanism by which asbestos induces tumors have been proposed (44). Since long, thin fibers are the most carcinogenic, it has been proposed that the asbestos fibers penetrate the lung and irritate the lining of the chest wall. The chronic inflammation and scarring would then contribute to tumor formation. Alternatively, the fibers may pierce spindle fibers during mitosis and thereby induce chromosome damage. Finally, asbestos fibers may induce oxidative stress and/or alter MAPK cell signaling. Significantly, epidemiologic studies show that cigarette smoking acts synergistically with asbestos exposure to induce lung tumors. In addition to asbestos, exposure to other fibers such as plant-derived silica fibers (biogenic silica) has been shown to be carcinogenic (45).

Hormones

The etiology of numerous cancers is believed to be influenced by hormonal or dietary factors, and hormones under certain conditions are considered to be known human carcinogens (Table 7-4). As previously mentioned, overweight and obesity are associated with elevated cancer risk. This effect may be mediated by endocrine dysregulation such as altered insulin and IGF-1 levels. Additionally, prostate, ovarian, breast, testicular, and endometrial cancers are hormonally driven (46–48). A role for hormones in cancer etiology was established when castration and ovariectomy studies revealed that hormone-dependent cancers could be prevented by removing the primary hormone-synthesis organs. As an example of the action of hormones in cancer formation, estrogen activates hormone-responsive receptors. Stimulation of these receptors, such as the estrogen receptors, can increase the cellular proliferation rate to promote tumorigenesis. Endogenously synthesized hormones and administered hormones have been shown to influence cancer formation. Hormone replacement therapy and estrogen-only birth control therapy have been associated with increased risk of hormone-dependent cancers. An even more dramatic example of synthetic hormone-induced cancer is that of women who were exposed to estrogenic diethylstilbestrol (DES) in utero. DES was taken by pregnant women to prevent abortion; however, a percentage of female offspring developed clear cell carcinomas of the vagina and cervix after the onset of puberty.

Mechanisms of Chemical Carcinogenesis

Multistage Nature of Carcinogenesis and the Multistage Model of Mouse Skin Carcinogenesis

The multistage nature of carcinogenesis has been appreciated for many years. The concept was developed when it was discovered that wounding of the skin of mice previously treated with mutagenic coal tar led to skin tumor formation. Since that time, the multistage conceptual model of cancer development has been

extensively studied and verified. The model holds that tumors arise in cells that have first undergone a mutating event. Subsequently, cell proliferative stimuli promote the initiated cell population to expand, resulting in premalignant clonal outgrowths. Finally, additional genetic alterations accumulate in these lesions, leading to development of a neoplasm that becomes invasive and ultimately metastatic.

Numerous animal models have been developed to study the multistep nature by which various epithelial and other tumors develop and progress (reviewed in [49]). In one of the best-characterized models, the mouse two-stage skin carcinogenesis model, a subcarcinogenic dose of a mutating agent is delivered (50). This is followed by multiple exposures to growth-promoting stimuli and the appearance of tumors on the mouse's back (Figure 7-4). This model has provided an excellent paradigm in which to examine the carcinogenic potential of environmental agents and has been used to reveal the mechanistic bases for environmental carcinogenesis.

Initiation and Mutational Theory of Carcinogenesis

During the first stage of multistage carcinogenesis, DNA mutations result as a consequence of electrophilic carcinogen exposure, oxidative damage to DNA, DNA strand breaks, or other DNA insults. Mutations are believed to occur in multipotent stem cells and are inherited by daughter cells. The concept that cancer arises as a result of damage to genetic material was first proposed at the turn of the twentieth century by Theodor Boveri. In the 1950s and 1960s, James and Elizabeth Miller, after observing that a wide variety of structurally diverse chemicals could induce cancer in animal models, suggested that chemical carcinogens required metabolic activation to electrophilic intermediates. These electrophilic

intermediates could then covalently adduct proteins, RNA or DNA. The term “electrophile theory of chemical carcinogenesis” was coined to describe their concept. The Millers’ work was supported by data reported in 1964 by Brooks and Lawly, which demonstrated that the degree of covalent binding of carcinogenic PAHs to DNA correlated with carcinogenic potential (51). Subsequently, many chemical carcinogens have been shown to bind and alter DNA integrity thereby inducing mutations. Carcinogens that alter DNA to induce cancer in this manner are referred to as genotoxic carcinogens.

DNA mutations occurring in proto-oncogenes and tumor suppressor genes are particularly critical to the initiation of carcinogenesis. These normal cellular genes are targeted during carcinogenesis and play critical roles in tumor formation. Proto-oncogene mutations are dominant, in that activation of a single copy of a proto-oncogene to an oncogene may be significant for carcinogenesis. Proto-oncogenes are discussed in greater detail in Chapter 2; however, a list of proto-oncogenes and the cancers with which they are associated is provided in Table 7-5. In contrast to proto-oncogenes, the normal cellular function of tumor suppressor genes is to negatively regulate cell growth. According to Knudson’s two-hit theory, tumor suppressor genes require that both copies of the gene be lost or inactivated because tumor suppressor mutations are recessive in nature. For instance, inheritance of one mutated copy of *p53* is not significant until the second copy is lost (“second hit”), resulting in loss of heterozygosity (LOH). Examples of tumor suppressor genes and associated cancers are also provided in Table 7-5.

In the multistage mouse skin carcinogenesis model, initiation occurs via application of a genotoxic carcinogen (e.g., *N*-methyl-*N*-nitro-*N*-nitrosoguanidine [MNNG], 7,12-dimethylbenz[*a*]

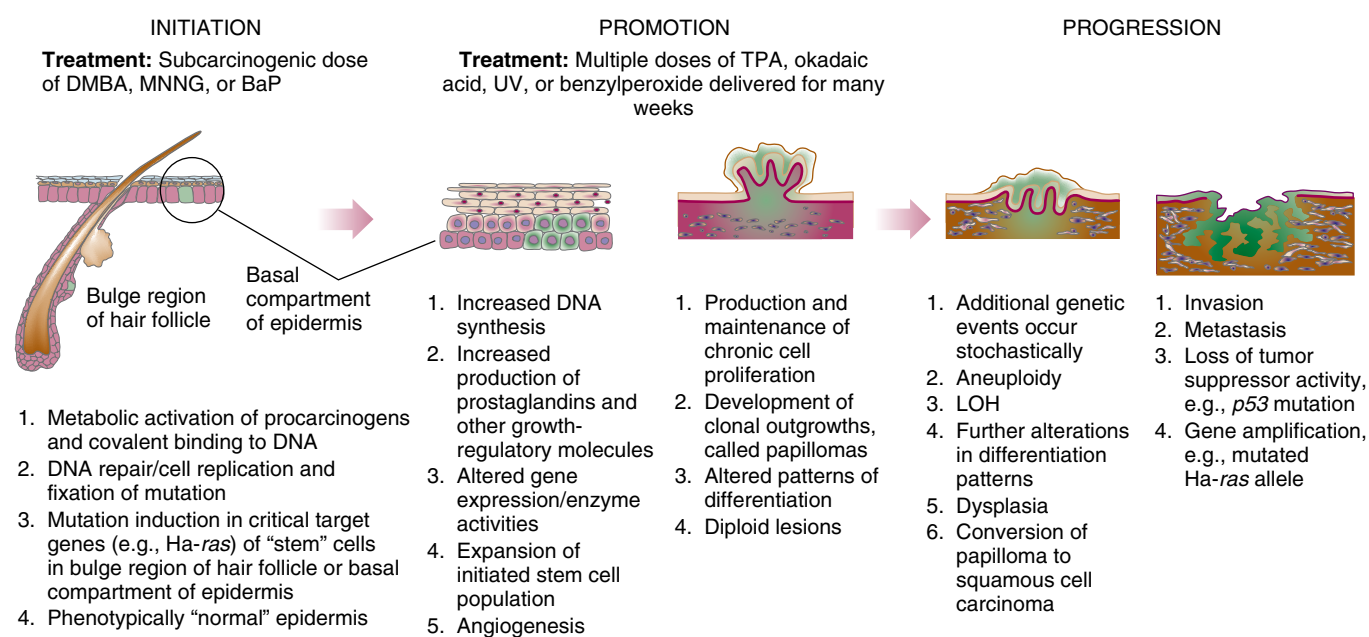


FIGURE 7-4 Multistage model of mouse skin carcinogenesis.

Table 7-5 Selected Proto-Oncogenes and Tumor Suppressor Genes and Some Associated Cancers^a

Gene Type	Gene Name	Associated Cancer Type
Proto-oncogene	<i>C-MYC</i>	Leukemia, lung, colon
	<i>ERBB-2</i>	Breast, ovary
	<i>ABL</i>	Leukemia
	<i>RASH</i>	Bladder
	<i>RASK</i>	Lung, ovary, bladder
	<i>RASN</i>	Breast
Tumor suppressor	<i>APC</i>	Colon
	<i>p53</i>	Breast, colon, lung
	<i>RB</i>	Retinoblastoma, breast, bladder, lung
	<i>BRCA1</i>	Breast, ovary
	<i>BRCA2</i>	Breast

^a Adapted from Perkins AS, Stern DF: Molecular Biology of Cancer. Oncogenes. In: DeVita VT, Hellman S, Rosenbert SA (eds.). *Cancer: Principles & Practice of Oncology*, 5th ed. Philadelphia: Lippincott-Raven Publishers, 1997.

anthracene [DMBA], B[a]P; Figure 7-4). A subcarcinogenic dose of the initiating agent is applied to the shaven dorsal skin of the mouse. The critical mutations for tumor development are believed to occur in epidermal multipotent stem cells, which may reside in the bulge region of the hair follicle as well as the basal compartment of the epidermis. The initiation stage is irreversible and cumulative. That is, the dose required for initiation can be divided and applied in portions over time or applied in a single dose with essentially the same result. Additionally, commencement of the promotion phase can be delayed since the DNA mutations induced by the initiating agent are permanent. In this model, the most frequently mutated proto-oncogene following initiation with PAH is *Ha-ras* (52). Mutations at G³⁸ of codon 13, in the case of B[a]P, and at A¹⁸² of codon 61, in the case of DMBA, activate *Ha-ras*. These mutations can be detected in the resulting tumors, reflecting the clonal origin of the papillomas. In addition, the specificity of these mutations is directly related to the major sites of DNA adduct formation arising from the carcinogenic diol epoxides of these two PAH carcinogens. In a variety of rodent models of multistage cancer (rat azoxymethane-induced colonic lesions, mouse diethylnitrosamine-induced liver foci, mouse urethane-induced lung adenomas) mutations in *ras* oncogenes frequently occur, highlighting the importance of *ras*, and oncogenes in general, in the development of cancer. Findings derived using these models have established the irreversible and cumulative nature of this stage of carcinogenesis and underscore the specificity of critical DNA mutations in proto-oncogenes or tumor suppressors induced by genotoxic carcinogens.

During carcinogenesis, numerous types of DNA lesions occur following exposure to carcinogenic agents. For example, in the case of electrophilic carcinogen attack, specific points within the DNA nucleotides are targeted for adduction (Figure 7-3). As noted previously, B[a]P targets the N² exocyclic amino group of guanine while other PAHs may target adenine in addition to guanine. As shown in Figure 7-3, alkylating agents target numerous sites within DNA bases. However, certain sites (e.g., O⁶ methylguanine and O⁴ methylthymine for methylating agents) may be the most important for carcinogenesis by this class of carcinogen. During

replication, mispairing due to DNA adducts or other DNA lesions may become fixed, and the ultimate effect depends on location of the mutation. Mutations affect coding sequences, intronic signals, untranslated regions, or promoter elements; consequently, protein function or expression levels may be altered. Following DNA double-strand breaks, incorrect rejoining of DNA has been shown to cause rearrangement of DNA coding and promoter regions. In addition to these qualitative changes, quantitative changes in gene copy number (gene amplification or gene deletion) may also affect key cancer-associated genes.

Promotion

During promotion, epigenetic changes allow for clonal expansion of initiated cells. This stage is characterized by altered gene expression and proliferation of initiated cells. Most tumor promoters are thought to exert their effects through cellular receptors or cell growth, differentiation, and/or apoptotic signaling pathways. Since promoting agents do not exert their effects via a direct effect on DNA, their actions are believed to occur through epigenetic mechanisms. Tumor promoters are thought to work via a variety of reversible mechanisms such as inducing chronic cell injury, immunosuppression, or inappropriate activation of cellular receptors.

In the mouse two-stage skin carcinogenesis model, the promotion stage is elicited by multiple applications of promoting agents delivered over the course of weeks or months (Figure 7-4; 53). In this model, promotion must occur following initiation with a mutating agent. As opposed to initiation, the promotion stage is initially reversible, does not elicit DNA mutation, is prolonged in nature, and appears to be nonadditive. Typical skin tumor-promoting agents include the phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA); the phosphatase inhibitor, okadaic acid; and the organic peroxide, benzoyl peroxide. Additionally, UV light, repeated abrasion, and certain silica fibers possess the ability to function as tumor promoters. The end point of promotion in the mouse two-stage skin carcinogenesis model is the development of premalignant, clonal outgrowths referred to as squamous papillomas. These lesions (hyperplastic epidermis folded over a core of stroma) are still well-differentiated and do not possess the ability to invade surrounding tissue. Once the cells of the papilloma acquire additional mutations that allow autonomous growth, the promotion stage is no longer reversible.

Promoting agents act via an epigenetic mechanism to alter gene expression (54). The initial interaction of promoting agent with the cell depends on the nature of the promoter. In the mouse skin model, the receptor for TPA (the most frequently used promoting agent) has been identified as protein kinase C (PKC). Stimulation of PKC results in a cascade of events that allow for expansion of the initiated cell population. PKC-mediated signaling events include induction of ornithine decarboxylase (ODC) activity, activation of the MAPK pathway, and up-regulation of ligands for the EGFR. Downstream of MAPK and EGFR activation, proliferative processes are stimulated. For instance, Akt and Stat3 signaling are believed to affect cell-cycle parameters via altered cyclin D1 expression (55,56). While the initial mechanism for other skin tumor promoters (e.g., okadaic acid, benzoyl

peroxide) is different from that of TPA, all tumors promoters ultimately elicit key biologic and molecular changes. These changes include induction of ODC, induction of growth factors and cytokines, production of eicosanoids, and increased DNA synthesis. Growth factors and cytokines known to be altered by tumor-promoting stimuli include TGF- α , TGF- β , IL-1, IL-6, and TNF- α among others.

Tumor-promoting agents have been identified for a number of rodent tissues other than mouse skin, indicating the generality of this phenomenon to other organs and species (reviewed in [49]). Some examples of tumor promoters that act on organs other than skin include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (liver), butylated hydroxyanisole (BHA) (lung), sodium saccharin (urinary bladder), and bile acids (colon).

Progression

The final stage of carcinogenesis is referred to as progression. The original tumor mass increases in size, additional mutations accumulate, and invasion and metastasis occur. Crucial to progression of solid tumors is the ability of cancer cells to invade the surrounding stroma, enter the bloodstream, and extravasate to colonize distal sites. For cells to break away from the primary tumor, down-regulation of cell adhesion, often by repression of E-cadherin expression, must occur. Subsequently, the cells must acquire mobility and ability to invade the surrounding stroma and blood vessel basement membranes. Invasion requires the action of degradation enzymes such as the matrix metalloproteinases (MMPs). Once established, the metastatic colony must develop adequate nutrition and oxygen supply via stimulation of angiogenesis.

During progression in the two-stage mouse skin carcinogenesis model, premalignant papillomas convert to SCCs (Figure 7-4; 57). This conversion process is accompanied by additional genetic alterations, including development of aneuploidy. Characteristic changes in gene expression such as elevation in gamma-glutamyltranspeptidase, $\alpha_6\beta_4$ integrin and keratin-13 expression as well as loss of E-cadherin expression are also common. The histologic appearance of SCCs can be distinguished from papillomas by downward growth and loss of ordered differentiation of epidermal keratinocytes.

Multistage Environmental Carcinogenesis in Humans

The applicability of multistage carcinogenesis concepts to human cancer is supported by a number of observations. First, human environmental carcinogen exposure outside of occupational settings usually occurs in low doses repeatedly delivered over the course of months or years. Each individual dose alone is likely insufficient to produce cancer. Additionally, it is unlikely that a single dose of a sole agent is the cause of most human cancers. Second, there is considerable evidence from human epidemiologic and experimental animal studies that certain human carcinogens such as tobacco smoke and UV light exhibit a strong tumor-promoting activity. Furthermore, many components of the human diet appear to influence cancer in humans through a tumor promotion type of

effect. Finally, histochemical and molecular examination of tumors at various stages indicates that human cancers develop via multiple steps. It has been postulated that human cancers require as many as four to six sequential genetic events for their development (58).

Numerous human cancers, particularly those of epithelial origin, appear to develop in a multistage progression. For instance, regions of dysplasia and carcinoma *in situ* appear to precede invasive carcinoma when melanoma, head and neck squamous cell carcinoma, and cervical cancer lesions are examined. Supporting the multistage nature of cancer development, genetic alterations have been shown to accumulate during tumorigenesis in these lesions. For example, during colorectal carcinogenesis, mutations in the adenomatous polyposis coli (APC) gene appear to initiate tumorigenesis (59). A portion of the resulting dysplastic foci further accumulate mutations in the K-ras oncogene and other oncogene and tumor suppressor genes, and progress from adenomas to invasive carcinomas. A similar pattern of accumulation of molecular abnormalities has been noted for squamous cell lung carcinoma. As the severity of the histopathologic appearance of these lesions increases, the frequency of loss of heterozygosity events also increases (60).

Examination of tumor DNA has also validated a role for environmental carcinogens in the etiology of human cancer (61). When tumor suppressor and oncogene gene sequences are examined, characteristic mutation spectrums can be identified following carcinogen exposure. The mutation spectrum of the p53 tumor suppressor gene has been intensively studied. A database of over 10,000 reports of p53 mutations in human cancers has been collected. Depending on the cancer type, mutations are frequently reported at amino acids 130–142, 151–164, 171–181, 193–200, 213–223, 234–258, and 270–286, which are part of the DNA-binding domain of p53. Sixty-one percent of lung cancer samples have mutations at codon 157 in addition to mutations in codons 248 and 273. A large percentage of these mutations are the result of G→T transversions. *In vitro* analyses indicate that exposure of normal human bronchiolar epithelial cells to benzo[a]pyrene diol epoxide results in p53 adducts in the same mutation hot spots as in lung cancer: codons 157, 248, and 273 (62). These results provide strong evidence for a link between chemical carcinogen exposure [B[a]P of cigarette smoke] and human cancer (lung cancer; Figure 7-5). Additionally, aflatoxin B₁ exposure correlates strongly with liver cancer and p53 mutation at codon 249, whereas sunlight exposure, which is known to induce CC→TT transition mutations, correlates with CC→TT tandem mutations at hot spots for skin cancer in p53 (Figure 7-5).

Ecogenetics and Cancer Risk

Genetic Variation and Carcinogenesis

Various genetically influenced factors determine the ultimate outcome following environmental carcinogen exposure. Biotransformation of carcinogens, DNA repair, and cell signaling

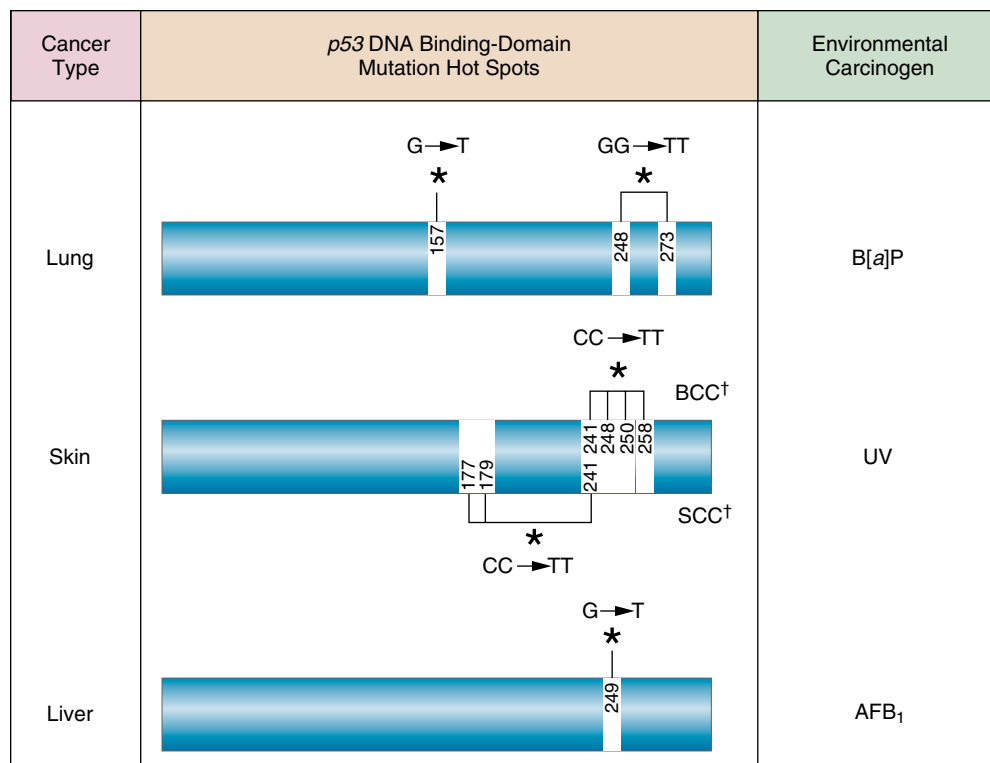


FIGURE 7-5 The *p53* mutation spectrum links cancer to environmental carcinogens. B[a]P has been shown to induce mutations in codons 157, 248, and 273 of the DNA binding domain of *p53*. Mutations in these codons are characteristic of the mutation spectrum found in lung cancer samples. Additionally, mutations in codon 249 are noted in liver cancers and are associated with AFB₁ exposure, while ultraviolet light induces CC→TT mutations detected in basal cell carcinoma and squamous cell carcinoma samples.

†Includes both aggressive and non-aggressive SCC and BCC.

pathways all play roles in response to environmental carcinogen exposure. Genetic variation within the human population in these classes of genes has been described and is expected, in some cases, to alter cancer risk. This interaction between genes and environment is referred to as ecogenetics (63). Research in the field of ecogenetics seeks to describe the relationship between toxicant exposure outcome and the mediating effects of genetic polymorphisms. Genetic polymorphisms are defined as genetic variations occurring with 1% or greater prevalence in a human population. Polymorphisms can occur in the form of large deletions, small deletions, small insertions, and individual base changes, especially single-nucleotide polymorphisms (SNPs). These polymorphisms can occur in exons, introns, and promoter regions and demonstrate a wide variety of effects ranging from no effect to structural changes to expression level alterations and protein stability alterations. The physiologic relevance of genetic variation may be a function of the severity of the alteration and the level of carcinogen exposure. Thus, genetic variation may be most important in the context of low-level exposure since high-level carcinogen exposure may overwhelm any differences in effects that may result from variation in environmental exposure response genes. Although genetic variation can influence cell signaling, cellular differentiation, and cellular proliferation during chemical carcinogenesis, historically, more attention has been given to identification of genetic determinants of carcinogen metabolism and DNA repair.

Carcinogen Metabolism

Phase I and Phase II Biotransformation Reactions

Subsequent to absorption through the gastrointestinal tract, xenobiotics travel via the portal vein to the liver where “first-pass metabolism” occurs. Hepatic tissues are highly concentrated with metabolic enzymes specialized in chemical conversion referred to as biotransformation. In other cases, such as inhalation or dermal exposure, biotransformation enzymes at the site of exposure can begin immediately to convert parent compound into metabolites. Biotransformation enzymes are theorized to have evolved as natural defenses against environmental toxin exposure.

The reactions catalyzed by biotransformation enzymes have been categorized into groups referred to as “phase I” and “phase II” reactions due to their often sequential roles in conversion of xenobiotics. Phase I reactions include oxidation, reduction, and hydrolysis reactions and, generally, expose functional groups that enable phase II biotransformation to proceed. Phase II biotransformation reactions catalyze glucuronidation, sulfation, acetylation, methylation, and glutathione conjugation reactions, among others. Numerous enzymes that catalyze these reactions have been identified and classified according to gene family (Table 7-6).

Phase I metabolites, in general, display minimally increased hydrophilicity. In contrast, phase II biotransformation reactions catalyze addition of cofactor molecules to the parent compound,

Table 7-6 Selected Phase I and Phase II Biotransformation Enzymes

Enzyme Classification	Reaction Catalyzed	Gene Family	Class/Subfamily	Isoforms
Phase I	Oxidation, others	Cytochrome P450	CYP1	CYP1A1 CYP1A2 CYP1B1
			CYP2	CYP2A6 CYP2A13 CYP2B6 CYP2C8 CYP2C9 CYP2C19 CYP2D6 CYP2E1
			CYP3	CYP3A4 CYP3A5 CYP3A7 CYP3A43
			CYP4	CYP4A11 CYP4A22 CYP4B1
			CYP>4	CYP5A1 CYP8A1 CYP19A1 CYP21A2
	Hydrolysis	Epoxide hydrolase	Microsomal Cytosolic	EPHX1 EPHX2
Phase II	Glutathionylation	Glutathione S-transferase	Alpha	GSTA1 GSTA2 GSTA3 GSTA4 GSTA5
			Mu	GSTM1 GSTM2 GSTM3 GSTM4 GSTM5
			Omega	GSTO1 GSTO2
			Pi	GSTP1
			Theta	GSTT1 GSTT2
			Zeta	GSTZ1
	Acetylation	<i>N</i> -acetyltransferase		NAT1 NAT2
	Sulfation	Sulfotransferases	SULT1	SULT1A1 SULT1A2 SULT1A3 SULT1B1 SULT1C2 SULT1C4 SULT1E1
			SULT2	SULT2A1 SULT2B1
			SULT4	SULT4A1
	Methylation	Catechol- <i>O</i> -methyltransferase	Soluble Membrane-bound	S-COMT MB-COMT

Table 7-6 Selected Phase I and Phase II Biotransformation Enzymes—Continued

Enzyme Classification	Reaction Catalyzed	Gene Family	Class/Subfamily	Isoforms
	Glucuronidation	UDP-glucuronosyl transferases	UGT1	UGT1A1 UGT1A3 UGT1A4 UGT1A5 UGT1A6 UGT1A7 UGT1A8 UGT1A9 UGT1A10
			UGT2	UGT2A1 UGT2A2 UGT2B4 UGT2B7 UGT2B10 UGT2B15 UGT2B17 UGT2B28
			UGT3	UGT3A1 UGT3A2
			UGT8	UGT8A1

resulting in a significant increase in hydrophilicity. In certain instances, phase II conjugation reactions may also target the parent compound for export via specialized efflux pumps. Therefore, in general, phase II biotransformation reactions ultimately result in metabolites that are less toxic and more readily excreted. In contrast, phase I biotransformation of carcinogens often results in reactive metabolites capable of covalent modification of cellular macromolecules. It is important to note, however, that these are generalizations. Examples of phase I–mediated detoxification have been noted, and phase II–mediated chemical activation has been documented.

According to the Millers' electrophilic theory of carcinogenesis, all mutagenic compounds must be inherently chemically reactive or converted via biotransformation to a reactive form. Carcinogens that do not require metabolic activation are referred to as direct carcinogens; indirect carcinogens require metabolic activation. The conversion of parent compound to a reactive state converts a procarcinogen to an ultimate carcinogen. Ultimate carcinogens, like direct carcinogens, are electrophilic and attack nucleophilic groups in DNA to initiate carcinogenesis as discussed in the section titled "Initiation and Mutational Theory of Carcinogenesis." Although categorizing biotransformation reactions according to the "phase I" versus "phase II" nature of the metabolism is useful, the endpoint of carcinogen exposure is often determined by a combination of oxidation, reduction, and conjugation reactions.

PAH Biotransformation

PAHs are widely studied substrates for cytochrome P450 (CYP450)–mediated biotransformation. CYP450s, a class of enzymes present in the endoplasmic reticulum of most cells, have been implicated in numerous carcinogen activation reactions. In humans, the CYP450 family consists of more than 50 genes,

which are grouped on the basis of sequence similarity into families (1, 2, 3,...), subfamilies (A, B, C,...), and individual CYP450s (1, 2, 3,...) (e.g., CYP450 1A1, 1A2, 1B1, etc.) (64). CYP450s catalyze oxidation, reduction, oxygenation, dealkylation, desulfuration, dehalogenation, and hydroxylation reactions. CYP450-mediated reactions can detoxify direct carcinogens and activate indirect carcinogens.

Once absorbed, certain PAHs can be biotransformed into electrophilic mutagens via the sequential action of phase I enzymes (Figure 7-6; 23). First, PAH double-bond oxidation is catalyzed by CYP450 enzymes. For example, in the case of B[a]P, CYP450-mediated oxidation forms the epoxide intermediate, benzo[a]pyrene 7R,8S-epoxide. Next, microsomal epoxide hydrolase (MEH) catalyzes hydrolysis of arene oxide to a *trans* dihydrodiol. Finally, a CYP450-catalyzed oxidation reaction forms the ultimate carcinogen (i.e., benzo[a]pyrene 7,8 diol-9,10 epoxide), a diol-epoxide metabolite. In human lung tissue, both B[a]P epoxidation steps are catalyzed primarily by CYP1A1. PAHs can be detoxified by glutathione S-transferases (GSTs). GST-mediated glutathione conjugation of PAH epoxides can deactivate the ultimate carcinogen or prevent activation to reactive diol epoxides.

Aflatoxin Biotransformation

Metabolism plays a critical role in determining carcinogenicity of the mycotoxin, AFB₁. AFB₁ must first be activated to the ultimate carcinogen, exo-8,9-AFB₁-epoxide (Figure 7-6). This reaction is predominantly catalyzed by CYP450 3A4 in humans (25). Alternatively, CYP450s can metabolize AFB₁ to inactive products such as AFM₁, AFQ₁, or AFB₁ endo-8,9-epoxide (AFBO).

Glutathione conjugation catalyzed by GSTs plays a critical role in protecting against mutagenic and carcinogenic effects of

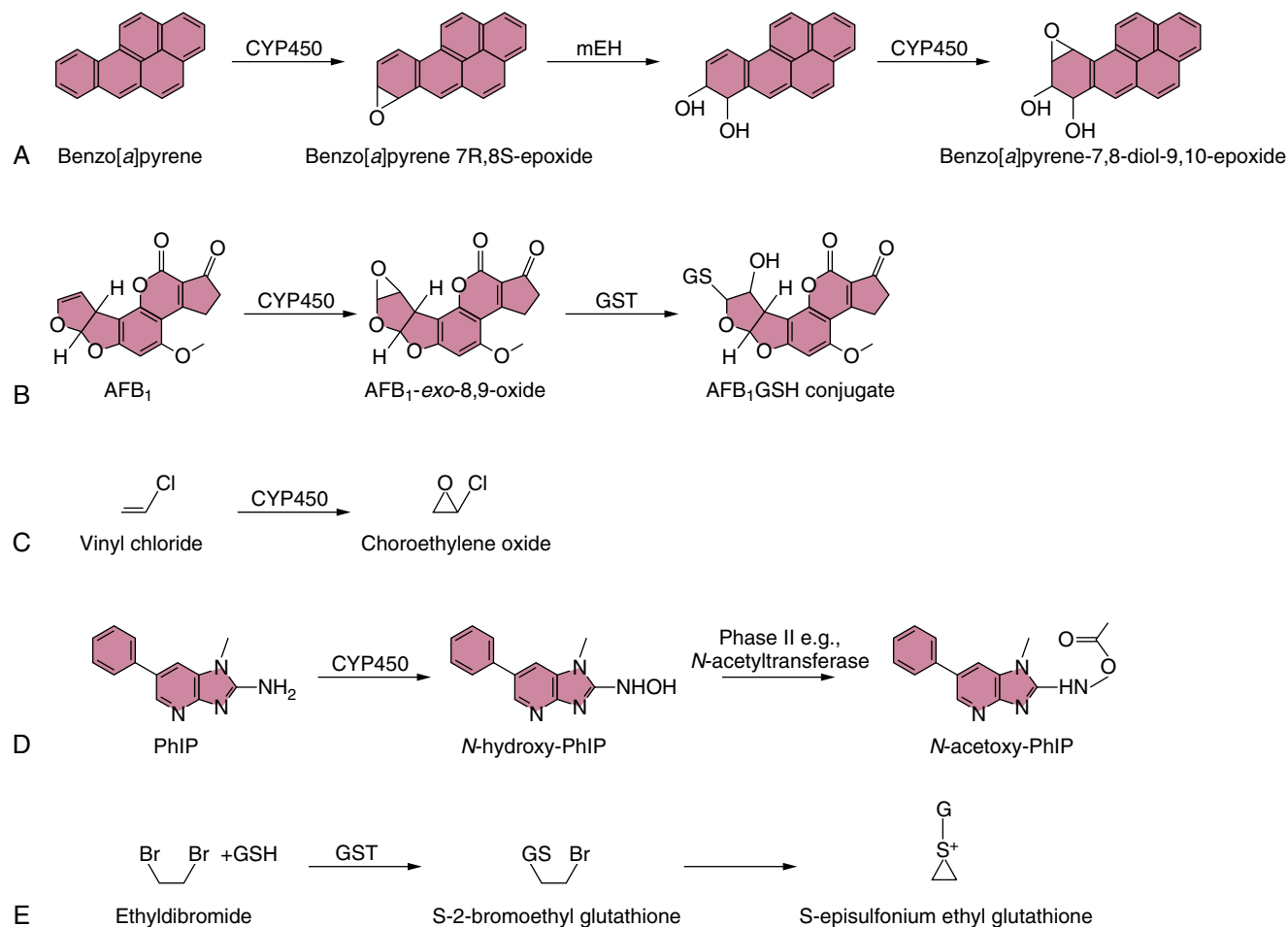


FIGURE 7-6 Biotransformation either activates or deactivates the ultimate carcinogen. **A:** Sequential action of CYP450 and mEH activates B[a]P. **B:** CYP450 activates while glutathione S-transferase (GST)-mediated GSH conjugation deactivates AFB₁. **C:** Vinyl chloride is activated to its epoxide metabolite by CYP450. **D:** The 2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine (PhIP) is first metabolized by CYP450 then activated by NAT. **E:** GST mediates activation of ethyldibromide.

AFB₁ metabolites. Generally, GSTs facilitate xenobiotic clearance by catalyzing glutathione conjugation of a variety of electrophilic substrates (65). In humans, cytosolic GSTs are categorized according to gene sequence similarity into at least six classes: Alpha (A), Mu (M), Omega (O), Pi (P), Theta (T), and Zeta (Z). Individual GST family members demonstrated unique, though overlapping substrate specificity.

Subsequent to activation, GST-mediated GSH conjugation can detoxify the AFB₁ epoxide, and this reaction is a major factor underlying the substantial species variation in sensitivity to AFB₁-induced carcinogenesis. For example, rats are highly sensitive to AFB₁-induced hepatocarcinogenesis, whereas mice are comparatively resistant. In line with this observation, mice express mGSTA3-3, which demonstrates high activity toward AFBO, whereas rat GST-mediated deactivation of AFB is drastically less in comparison. Mutational studies of recombinant mGSTA3-3 indicate that the high activity of this protein toward AFBO is due to multiple, critical amino acid residues in the substrate binding site that are not present in homologous rat GSTA3-3 (66).

Vinyl Chloride Biotransformation

Vinyl chloride is the starting material for production of polyvinyl chloride, used in fabrication of products such as PVC pipe. The mutagenicity of this liver carcinogen is dependent on metabolic activation by CYP450, and detoxification is mediated by microsomal epoxide hydrolase (mEH; 67). As shown in Figure 7-6, vinyl chloride is a relatively simple compound. In the presence of oxygen and NADPH, CYP450 2E1 catalyzes formation of a highly unstable epoxide moiety across the central carbon double bond. This epoxide, chloroethylene oxide, is the ultimate carcinogen capable of covalently adducting DNA. Chloroethylene oxide can be detoxified via the action of mEH as noted previously or by GST-mediated glutathione conjugation.

Benzidine Biotransformation

Benzidine, an aromatic amine bladder carcinogen, must also undergo metabolic activation to initiate carcinogenesis (29). The activation of benzidine via N-oxidation is catalyzed by CYP450 enzymes. Subsequent to N-oxidation, N-acetyltransferase

(NAT)–catalyzed *O*-acetylation forms electrophilic *N*-acetoxy derivatives capable of attacking DNA. In contrast, *N*-acetylation is also believed to compete with *N*-oxidation and, therefore, is considered a detoxification reaction when it occurs prior to formation of the *N*-OH metabolites. *N*-glucuronidation of oxidized benzidine catalyzed by UDP-glucuronosyltransferase (UGT) is a second detoxification mechanism, since *N*-glucuronidation facilitates excretion. Therefore, in the case of benzidine biotransformation, phase II reactions activate and detoxify the carcinogen.

Heterocyclic Amine Biotransformation

Heterocyclic amines, found in cooked meat and fish, are initially activated to genotoxic metabolites via CYP450-mediated oxidation to the *N*-hydroxyl derivative (Figure 7-6; 32). In particular, this reaction is catalyzed in the liver predominantly by CYP450 1A2 (CYP1A2). The hydroxylated heterocyclic amine metabolites are then further activated by acetyltransferases and sulfotransferases to the ultimate carcinogen, a highly reactive electrophile. GSTs and UDP-glucuronosyl transferases are thought to deactivate the ultimate carcinogen and permit elimination. Therefore, during the biotransformation of heterocyclic amines, phase II enzymes activate and detoxify the carcinogen.

Ethyldibromide Biotransformation

An additional example of phase II–mediated carcinogen activation is that of the halogenated aliphatic, ethyldibromide (68). Ethyldibromide is a potent mutagen used as an industrial solvent, gasoline lead scavenger, and fumigant. Following glutathione

conjugation of the parent compound, *S*-2-bromoethyl glutathione spontaneously forms an episulfonium ion (Figure 7-6). This sterically strained molecule is the reactive ultimate carcinogen and primarily attacks the N⁷ position of guanine. Again, although GSTs commonly detoxify xenobiotics, glutathione conjugation of ethyldibromide precedes carcinogen activation.

Biotransformation Enzyme Polymorphisms and Cancer Risk

Glutathione *S*-Transferase Polymorphisms

Numerous polymorphisms in the genes for biotransformation enzymes have been described and linked to altered metabolism of carcinogens (69). SNPs occur in GST gene exons, introns, and promoter regions, and two gene deletion polymorphisms have been described (70). The *GSTM1* and *GSTT1* genes are deleted in ~50% and ~20–60% of the population, respectively. Polymorphisms have also been described for CYP450, NAT, and *mEH* genes as shown in Table 7-7. A number of these alterations have been shown experimentally to alter either the expression level or catalytic activities of their corresponding proteins.

One of the most studied biotransformation enzyme/carcinogen ecogenetic relationships is the relationship between *GSTM1* deletion polymorphism and lung cancer risk. *GSTM1* detoxifies PAHs such as those in cigarette smoke, and meta-analysis of epidemiologic data suggests that *GSTM1* deficiency is a moderate risk factor for lung cancer (71). Similarly, *GSTM1* deletion may increase risk of colon and bladder cancers (72,73). However, some studies of *GSTM1* genotype and cancer phenotype have reported inconsistent outcomes; therefore the relative contribution of this

Table 7-7 Partial Listing of the Numerous Polymorphisms Identified in Human Biotransformation Enzyme Genes

Gene	Polymorphism Designation	Polymorphism	Effect
<i>GSTA1</i>	<i>GSTA1</i> *A, <i>GSTA1</i> *B	5' Promoter SNPs	Differential mRNA expression
<i>GSTM1</i>	<i>GSTM1</i> *o	Gene deletion	No protein produced
<i>GSTP1</i>	<i>GSTP1</i> *A, <i>GSTP1</i> *B, <i>GSTP1</i> *C, <i>GSTP1</i> *D	Ile104Val Ala113Val	Altered activity and substrate affinity
<i>GSTT1</i>	<i>GSTT1</i> *o	Gene deletion	No protein produced
<i>CYP1A1</i>	<i>CYP1A1</i> *2A	T3801C, Noncoding region SNP	Unknown effect
	<i>CYP1A2</i> *1F, <i>CYP1A2</i> *1K	5' Promoter/intronic SNPs	Differential mRNA expression/induction?
<i>CYP2A6</i>	<i>CYP2A6</i> *1B	3' UTR SNP	Stabilized mRNA
	<i>CYP2A6</i> *4	Gene deletion	No protein produced
	<i>CYP2A6</i> *9	5' Promoter SNP	Altered mRNA expression
<i>NAT2</i>	<i>NAT2</i> *5D, <i>NAT2</i> *6B, <i>NAT2</i> *7A, <i>NAT2</i> *10, <i>NAT2</i> *12A, <i>NAT2</i> *14A, <i>NAT2</i> *17, <i>NAT2</i> *18, <i>NAT2</i> *19, etc	Ile114Thr, Arg197Gln, Gly286Glu, Glu167Lys, Lys268Arg, Arg64Gln, Gln145Pro, Lys282Thr, Arg64Trp, etc.	Altered enzyme activity (Rapid vs. slow acetylator phenotype)
<i>mEH</i>		Tyr113His	Altered enzyme activity
		His139Arg	Altered enzyme activity

SNP, single-nucleotide polymorphism; UTR, untranslated region.

polymorphisms requires further investigation. Studies of *GSTM1* deletion polymorphism in the context of other carcinogen-response gene polymorphisms may be critical. Likewise other GST genotype–phenotype relationships have been investigated with varying degrees of consistency. For instance, GST α class protein expression levels have been correlated with colorectal cancer risk (74). *GSTT1* deletion polymorphism may increase risk of cancers of the head, neck, and oral cavity (75), and coding region polymorphisms in the *GSTP1* gene appear to confer breast cancer risk (76).

Cytochrome P450 Polymorphisms

CYP1B1, CYP1A1, CYP1A2, CYP2E1, and CYP3A4 have all been shown to participate in the biotransformation of procarcinogens to ultimate carcinogens as noted in the preceding section (and reviewed in 77). Interestingly, these genes are generally well conserved and few functionally relevant polymorphisms have been described (77,78). Studies using *CYP1B1* knockout mice highlight the important function of this enzyme in PAH activation. *CYP1B1*-deficient mice are resistant to DMBA-induced carcinogenesis, due to a lack of DMBA conversion from procarcinogen to ultimate carcinogen (79). However, few unequivocal examples of CYP450 polymorphism–modified cancer risk in the human population are available. This is believed to be due to environmental confounders, low frequency of polymorphisms, and inheritance of multiple genes that modify outcome. Regardless, *CYP2A6* genotype may affect tobacco-induced lung cancer risk, and a high *CYP1A1* inducibility/activity phenotype has been associated with elevated lung cancer risk, likely due to its role in activating B[a]P and possibly other PAHs in cigarette smoke (80–82). This effect is even more prominent in smokers who also inherit the *GSTM1* null genotype. However, the genetic basis for variation in *CYP1A1* activity has not been indisputably explained, although attempts to link the phenotype to polymorphisms in noncoding regions have been made.

N-acetyltransferase Polymorphisms

Phenotypic variation in acetylation catalyzed by *N*-acetyltransferase (NAT) was first discovered when interindividual variation in isoniazid sensitivity was described. This drug metabolism variation was eventually attributed to genetic variation in the *NAT2* gene. The molecular basis for “fast-acetylator” versus “slow-acetylator” status is complex due to inheritance of various combinations of *NAT2* SNPs affecting protein expression and catalytic activity (over 25 human *NAT2* alleles have been reported). Inheritance of *NAT2* polymorphisms has been linked to altered cancer risk, likely due to the role of acetylation in mediating aromatic and heterocyclic-amine carcinogenicity (83,84). The classical example of *NAT2* polymorphism and altered cancer risk is that of urinary bladder cancer risk and *NAT2* slow acetylator phenotype. Aromatic amines, such as those found in cigarette smoke, require activation via *N*-hydroxylation to become mutagenic. Hypothetically, *NAT2*-mediated *N*-acetylation competes with *N*-hydroxylation to prevent activation thereby explaining the observation that slow acetylators are at increased risk of urinary bladder cancer. In contrast, studies of colon cancer demonstrate, although inconsistently,

an elevation in cancer risk in fast acetylators. In theory, these findings can be mechanistically explained given that *N*-acetylation functions to further activate *N*-hydroxylated heterocyclic amines found in cooked meats; therefore, fast acetylators are expected to have elevated levels of highly reactive metabolites.

These studies illustrate the complex nature of predicting phenotype based on genotype when factors such as carcinogen dose and tissue-specific expression of biotransformation genes must be taken into account in the context of low-penetrance phenotypes. Additionally, it is well known that many dietary and environmental chemicals can alter expression or activity of phase I and phase II enzymes, further confounding the genotype–phenotype relationship. However, the ultimate goal is to achieve cancer risk modeling that takes into account both inheritance of polymorphisms in multiple genes in carcinogen biotransformation pathways as well as other confounding factors such as coincidental environmental exposures.

DNA Repair

DNA Repair Pathways

Various forms of carcinogen-induced DNA damage, such as DNA adducts, DNA cross-links, and double- and single-strand breaks, have been reported. To maintain genomic integrity, DNA repair genes have evolved (85). Over 125 DNA repair enzymes and DNA damage response genes have been identified. The importance of these genes is highlighted by inherited syndromes (e.g., xeroderma pigmentosum [XP], Fanconi anemia, Bloom syndrome, and ataxia telangiectasia) wherein DNA repair defects render the individual highly susceptible to cancer incidence. These DNA repair proteins can be generally categorized according to the repair pathways in which they function or according to their ability to signal for or regulate DNA repair (see Chapter 4). The predominant human DNA repair pathways include base excision, nucleotide excision, base mismatch, and DNA strand break repair. Simpler, direct repair pathways have also been reported.

DNA Repair Gene Polymorphisms and Cancer Risk

Polymorphisms in DNA repair genes may obliterate or compromise the function of the pathways in which they participate (86). Ultimately, these polymorphisms may decrease DNA damage repair efficiency, increase the mutation rate, and elevate cancer risk in carriers of the DNA sequence alterations. High-penetrance genetic alterations such as are inherited in disorders such as XP illustrate this point. Patients with XP have greatly reduced capacity to carry out nucleotide excision repair (NER); therefore, they are very sensitive to damage by UV and are at high risk for skin cancer. High-penetrance alterations such as these are relatively rare. More commonly, polymorphisms with low-penetrance effects are detected and alter response to carcinogen exposure. For example, polymorphisms in the base excision repair (BER) glycosylase/AP-lyase gene, *OGG1*, and in the NER XP genes, *XPA*, *XPB*, *XPC*, and *XPB*, have been noted and may affect cancer risk (86–89). In the case of *OGG1*, a relatively common polymorphism at codon 326 has been described. A C→G transversion converts serine 326 to a

cysteine residue with an allele frequency of approximately 20% to 40%. Since *OGG1* catalyzes removal of 8-oxoguanine from DNA, impaired function could be expected to alter mutation rate following oxidative insult. Indeed, in six epidemiologic studies elevated risk of esophageal, lung, prostate, or stomach cancer was noted. Additionally, two polymorphisms ($-77T>C$ and $R194W$) in the BER and single-strand break repair gene, *XRCC1*, have been linked to altered risk of lung and head and neck cancers (90–92). These studies highlight the relevance of ecogenetic relationships following carcinogen exposure.

Cancer Prevention

Since a significant fraction of cancer risk appears to be attributable to environmental factors, cancer prevention should be an attainable goal. Multiple approaches to cancer prevention have been proposed and include chemoprevention and, more simply, exposure reduction. As new products and pollutants are introduced into the environment, vigilance in hazard identification should largely prevent population-wide health crises such as those that led to the discovery of many occupational carcinogens in the 1970s and earlier. Additionally, careful analysis of current dietary and other environmental exposures will increase understanding of existing hazards. Furthermore, understanding of the underlying molecular mechanisms associated with the carcinogenic process will allow for design of effective chemoprevention strategies.

Hazard Identification

IN VITRO Assays

An important form of cancer prevention is hazard identification. To effectively prevent human exposure to carcinogens, the carcinogen must be recognized as such. Hazard identification occurs via multiple avenues under the direction of numerous institutes. Academic institutes, corporations, and government agencies all contribute to the identification of carcinogenic agents. Initial screening is often conducted using short-term, *in vitro* techniques. Several widely used assays have been developed and measure the mutagenicity of suspected carcinogens.

Ames Assay

The Ames assay of mutagenicity utilizes *Salmonella typhimurium* bacterial strains with unique growth requirements to detect mutagenicity of test compounds (93). In these assays, histidine-synthesis deficient *Salmonella* strains are initially grown in the presence of exogenous histidine and are subsequently exposed to test compounds. Mutations in histidine synthesis genes revert the bacterial strain to a histidine-independent status, which can be detected by growth in minimal-histidine media. Only those bacteria that have undergone mutation in histidine-synthesis genes are able to form colonies. Since bacterial strains cannot activate procarcinogens via CYP450 biotransformation, inclusion of mammalian metabolic enzymes is an important feature of this “reversion” assay.

HPRT Assay

The hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay uses cultured human somatic cells to detect mutagenic agents. The normal function of HPRT in cells is to recycle nucleotide bases from degraded DNA. To detect mutations in the *HPRT* gene, cells are first exposed to the test compound and then exposed to a toxic nucleotide analogue, 6-thioguanine (6TG). When HPRT is nonmutated and functioning, 6TG is incorporated into DNA, triggering cell death. However, when HPRT is inactivated by mutations, no 6TG is incorporated, and the cells live. Therefore, the number of surviving cells after a defined period of cell growth following 6TG exposure reflects the mutagenicity of the test agent.

Additional In vitro Carcinogen Identification Assays

In addition to the HPRT and Ames assays, several other direct and indirect *in vitro* assays for detection of genetic damage have been developed. Assays of changes at the chromosomal level in human cells include (1) the chromosome aberration assay, wherein metaphase chromosomes are examined for abnormalities; (2) the sister chromatid exchange (SCE) assay, wherein exchanges of identical pieces of chromosomes in duplicated sister chromatids are examined in metaphase cells; and (3) the micronucleus assay, wherein the number of chromosome fragments referred to as micronuclei are counted.

In Vivo Assays

Two-year bioassays in rodents are currently used extensively for carcinogen identification. Whole animal assays are conducted to determine the carcinogenic potential of agents when delivered over the life span in a more physiologically relevant model. Of the approximately 200 agents classified as human carcinogens, almost all have been shown to cause cancer in rats or mice, highlighting the utility of animal studies in identification of carcinogens. Rodents are administered the test compound via the exposure route most relevant to human scenarios at two doses: the maximum tolerated dose (MTD) and one half the MTD. The compound is administered for a majority of the life span of the animal, and tumor incidence at all sites is recorded. Generally, the rat is recommended for the first 2-year carcinogenicity study. These data are then supplemented with additional short- or medium-term *in vivo* studies or with a 2-year carcinogenicity study in another rodent species such as the mouse. Short- and medium-term testing may include the use of transgenic or “knock-out” mouse models wherein an oncogene is overexpressed or a tumor suppressor gene allele is missing, although the validity of using these genetically-altered models is still under debate.

Nongenotoxic Carcinogens

Identification and analysis of so called “nongenotoxic” carcinogens is less straightforward than that for genotoxic agents. These agents are identified in the context of the 2-year rodent bioassay. Agents that are identified as carcinogenic in these *in vivo* assays but do not directly interact with DNA are classified as nongenotoxic

carcinogens (94). These agents characteristically induce tumors in only one or a few species and only after a threshold dose is achieved. Nongenotoxic carcinogens are not detected in *in vitro* assays of mutagenicity such as the Ames or HPRT assays. Many of these nongenotoxic carcinogens possess properties similar to tumor promoters suggesting, together with a lack of genotoxicity, that they work mechanistically differently than classical, genotoxic carcinogens. Considerable debate is ongoing concerning the best way to regulate such compounds (see subsequent sections).

Risk Assessment and Regulation of Carcinogen Exposure

As carcinogen exposure scenarios are identified, assessment of associated cancer risk, which considers predicted exposure and degree of health hazard, can be performed to determine when and if behavior modifications should be enforced. This process of predicting cancer risk in a given exposure scenario is referred to as risk assessment, whereas the response to predicted risk is referred to as risk management. Risk assessment concerning carcinogen exposure assumes, in contrast to other toxicant types (for example neurotoxicants), that no threshold dose exists. That is, no safe exposure level can be identified since any exposure dose could, in theory, induce a mutation in a critical target gene, thereby elevating cancer risk. This practice stands in contradiction to what is known about nongenotoxic carcinogens. Since these compounds often act in a species-specific manner and demonstrate a dose threshold, guidelines for risk assessment have been more complex to define (94). Extrapolation of a safe level of human exposure based on rodent data requires multiple assumptions. For instance, it is assumed that a rodent nongenotoxic carcinogen would be toxic to humans and that the no observable adverse effect level (NOAEL) in rodents could be applied to humans. Such decisions are greatly enhanced by mechanistic information so that judgments can be made concerning potential threat to human health. Currently, nongenotoxic carcinogens are regulated in the same manner as genotoxic carcinogens; however, study and debate continue. Much attention is also given to risk assessment at low doses to determine the health effects of chronic, low-level carcinogen exposure. The EPA is responsible for risk assessment in areas of known or suspected exposure of the population to carcinogens and makes recommendations for risk management to minimize health consequences due to environmental contamination. Both of the previously mentioned areas of debate bear great influence on risk management decisions and the clean-up goals set for areas of contamination.

Prevention Strategies

The goal of risk assessment and risk management is to prevent cancer by anticipating and circumventing carcinogen exposure. However, the etiology of certain cancers is still unknown. In many cases, risk assessment is impossible or risk management measures are unavailable. Furthermore, some carcinogen exposures are unavoidable or avoidance is not practically feasible. For instance, therapeutic radiation and certain chemotherapy drugs are known carcinogens; however, the risk-to-benefit ratio still favors voluntary

exposure, despite health risk. In these instances, prevention tactics are needed to counteract the carcinogenic process, especially in the absence of effective treatment options. Several approaches to prevention have been taken in recent years with varying degrees of promise.

Vaccination

Vaccination is among the most promising of approaches for biologic carcinogens such as human papilloma virus (HPV) and *Helicobacter pylori* (95). Development of vaccines to block initial infection with carcinogenic bacteria or virus would presumably prevent or reduce associated cancers. As an example, HPV vaccines have been developed to limit the spread of the virus and reduce the incidence of cervical cancer. In addition to this traditional use of vaccination, the use of vaccines against oncoantigens has also been proposed to prevent cancer via stimulating immune mechanisms to attack small cancerous lesions. Oncoantigens, which are tumor-associated molecules, are used to stimulate persistent immune memory mechanisms. When the antigen is later detected via immune surveillance, an effective adaptive immune response is mounted. In theory, the immune system is primed to detect and destroy any cancer cells expressing the oncoantigen. Success of vaccines in the prevention of tumors in animal models has been documented; however, the utility of such vaccines to prevent human tumors must still be validated.

Chemoprevention

Chemoprevention strategies for cancer incidence reduction have also been proposed. For instance, chemicals that up-regulate biotransformation enzymes (in particular, phase II enzymes) have been investigated as chemopreventative agents (96,97). Since most phase II biotransformation reactions reduce chemical reactivity of the parent compound, the rationale for inducing phase II enzymes or their cofactors is to reduce mutagenicity of initiating agents. For instance, oltipraz administration has been shown to attenuate AFB₁ toxicity in rats. Oltipraz elevates GST activity likely via activation of antioxidant response elements within GST promoter regions. Oltipraz may also inhibit the activation of aflatoxin by CYP450. The challenge associated with enzyme induction as a chemopreventative approach is that not all phase II biotransformation reactions are detoxification reactions. Since humans are exposed to a wide variety of carcinogens, induction of biotransformation enzymes may be simultaneously beneficial and detrimental. Therefore, the use of such chemopreventative agents must weigh multiple factors such as carcinogen target organ, agent distribution, and exposure scenario.

In addition to detoxification enzyme inducers, agents that combat or prevent oxidative stress are potential chemopreventative agents (98). Oxidative stress is believed to contribute to the formation of multiple cancer types; consequently, treatment with antioxidant agents may block carcinogenesis. In this regard, selenium, vitamin E, and lycopene are potent antioxidants under study for chemopreventative properties. Similarly, inflammation is believed to contribute to formation of numerous cancer types. Agents such as cyclooxygenase-2 (COX-2) inhibitors

(i.e., indomethacin) as well as other nonsteroidal anti-inflammatory drugs (NSAIDs) have been proposed as chemopreventative agents to combat procarcinogenic inflammation. Additionally, hormonal agents have been proposed for chemoprevention of cancers of reproductive organs such as breast and prostate cancers (99). For instance, selective estrogen receptor modulators (SERMs) have been proposed to prevent breast cancer by blocking the action of pro-carcinogenic estrogen. Tamoxifen, an anti-estrogenic agent, was first approved for treatment of advanced breast cancer but also reduced the risk of contralateral breast cancer occurrence. Tamoxifen has since been approved as a chemopreventive agent in high-risk patients.

Summary and Conclusions

Cancer is known to develop over many years and is determined by the interaction of host genetic factors as well as environmental exposures. Environmental factors appear to play a major role in determining cancer risk. Of the known cancer risk factors, smoking and diet account for a significant proportion of cancer deaths. There are many types of environmental carcinogens including biologic agents (e.g., viruses), chemicals (e.g., PAH), and physical agents (e.g., solar radiation). The linkage between environmental exposure and cancer in humans is strong in some cases

(e.g., asbestos and mesothelioma of the lung) whereas in other cases the environmental etiologic factors are less well understood (e.g., breast and prostate cancers). Epidemiologic studies, together with studies in model systems, especially animal model systems, provide the evidence used to determine the relative risk of specific environmental exposures. The process of categorizing cancer risk from environmental agents is an ongoing process conducted by the NTP and the IARC. Study of genetic polymorphisms in various genes involved in the carcinogenic process is leading to a better understanding of the overall risk associated with environmental exposures and identification of high-risk populations to target prevention strategies. Research on the underlying mechanisms associated with environmental carcinogenesis provides the basis for early detection and identification of target molecules for chemoprevention and/or intervention strategies. Carcinogens are known to target oncogenes and tumor suppressor genes through DNA damage and/or to alter cellular signaling pathways in bringing about the changes associated with cancer formation in specific tissues. Ultimately, environmental carcinogenesis occurs via the stepwise accumulation of genetic alterations leading to invasive and metastatic lesions. Finally, although many regulatory mechanisms exist to protect the public, diligence is required to safeguard from future unintended carcinogen exposures. It will remain prudent to closely monitor the environment for potential human carcinogens.

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Animal Models Flies, Fish, and Yeast

The molecular pathogenesis of human cancer is a complex process that often requires the cooperation of genetic mutations within many cellular pathways, ultimately leading to tumorigenesis. Simple model organisms with conserved genes and developmental pathways offer systems with which to dissect the role of individual genes and their contribution to the development of cancer in vivo. From single-celled yeasts to vertebrate fish such as the zebrafish, each model system provides its own unique strengths with which to identify new genes and to elucidate genetic interactions required for the development of cancer.

Why Use a Simple Model Organism?

Cancer develops as a result of disruption of the normal physiologic processes of cell growth, differentiation, and proliferation. Genes involved in these processes encode transcription factors and other regulatory proteins controlling the cell cycle, apoptosis, and survival. Protein involved in the highly conserved DNA repair apparatus are also mutated in ways that promote genomic instability. Even in the simplest eukaryotes many of these genes are conserved with higher species. Over several decades, the development of tools for forward genetic analysis based on phenotype in simple organisms has led to yeasts (*Saccharomyces cerevisiae*), the fruit fly (*Drosophila melanogaster*), and more recently the zebrafish (*Danio rerio*) emerging as the key simple organisms for investigating cancer genetics (Figure 8-1).

In addition to their individual strengths for investigating conserved pathways, there are three main practical reasons to use a simple model organism; time, space, and tractability. Yeasts such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Saccharomyces pombe* (*S. pombe*) are single-cell organisms. They have comparatively few genes and little redundant DNA in the form of introns. They replicate rapidly by budding (*S. cerevisiae*) or fission (*S. pombe*) and can be maintained in large numbers in both haploid and diploid states, facilitating the isolation and investigation of recessive mutations. Yeast cell numbers double every 100 minutes (given adequate nutrition), and these organisms are safe and inexpensive to maintain. The whole organism can be readily visualized by light microscopy and the incorporation of fluorescent proteins

allows subcellular localization of specific proteins in real time. Similarly *D. melanogaster* has a life cycle of 10 days, and large numbers of animals can be maintained in a small space. This multicellular organism can be used to examine cell–cell interactions and the roles of non-cell autonomous gene function in the development of cancer. Although *D. melanogaster* does not develop cancer in its classical form and lacks the closed blood system of vertebrates, ingenious genetic techniques have been applied to model pathways involved in the development of cancer and invasive metastases in this organism, which has the added advantage of being both multicellular and tractable to single-cell resolution (1). The zebrafish is a relatively new to the field of well-characterized model organisms, but has rapidly gained popularity. As a vertebrate with a closed vascular system (and beating heart), it provides an ideal intermediate model system between studies in invertebrates and small mammals such as the mouse. In contrast to the mouse, zebrafish development occurs rapidly outside the mother in transparent embryos, allowing direct visualization of the developing system and easy analysis of incorporated fluorescent markers. Its small size and high fecundity allow it to be easily maintained. The ability to directly visualize development in the embryo is particularly beneficial given that proto-oncogenes often have crucial roles during embryonic development.

Genetic Conservation and Synteny

Regulation of cell division is highly conserved in eukaryotes and has been extensively studied in yeasts. Conservation of a given gene through evolution is teleologically suggestive that the gene performs an essential function that can facilitate the investigation of genes and genetic pathways relevant to human cancer (and other diseases). In keeping with their essential roles, conserved genes are more likely to produce an embryonic lethal phenotype when disrupted in the homozygous state. This genotype–phenotype relationship in developmental biology is at the core of the investigative power of simple model organisms (Figure 8-2). Even in organisms where physiologic and evolutionary diversity suggest the presence of genes carrying out homologous functions would be very unlikely, interesting pathways are used for different functions, such

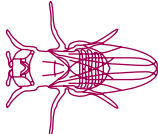
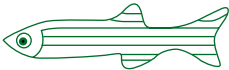
		
SACCHAROMYCES CERIVISAE	DROSOPHILA MELANOGASTER	DANIO RERIO
~7000 genes ~12 million bases	~15,000 genes ~132 million bases	~20,000 genes ~1547 million bases
Key features	Key features	Key features
• Single cell • Viable as haploid • Phenotypic variation during cell cycle • Use of selectable markers • Ease of plasmid insertion and homologous recombination • Rapid replication and large cell number • Few genes and little intronic DNA • Sequenced genome	• Multicellular but discernable at the single-cell level • Rapid development • Sequenced genome • Short life cycle • “Tumor” development • Easy assessment of non-cell autonomous function • Polytene chromosomes • Sequenced genome	• Vertebrate • Transparent embryos • Large numbers of progeny • Rapid ex-utero development • Develop true human-like cancers • Morpholino technology for gene knockdown • Transgenesis is well developed

FIGURE 8-1 Model organisms (the budding yeast, *Saccharomyces cerevisiae*; the fruit fly, *Drosophila melanogaster*; and the zebrafish, *Danio rerio*) and their key attributes as models for dissecting the molecular basis of cancer.

as the role of the *drosophila wingless* gene in larval segmentation and its human orthologue in mammalian brain development. The investigation of genetic interactions and signaling mechanisms of genes such as *wingless* in primitive developmental processes can still provide crucial information about its roles in mammalian development and cancer.

Developmental biology of simple organisms has shown not only that critical genes are conserved, and that such conserved genes are often essential during embryonic development, but also that genes required to direct cell fate, orientation, and differentiation are frequently proto-oncogenes or tumor suppressors, and that when such genes are mutated or misexpressed, this can lead to tumor development. Nevertheless, use of more evolutionarily complex organisms, such as the zebrafish allows examination of genetic pathways more closely resembling those that give rise to cancer in humans. However, the increased number of potentially redundant genes and introns in such organisms can make genetic analysis more complex than yeast or *Drosophila*. In teleost fish such as the zebrafish, there are often two or three copies (paralogs) of individual genes. This is a result of genome duplication that likely occurred more than 150 million years ago (2). Several methods permit determination of which copy of such genes is functionally homologous to the human gene. The analysis of genetic synteny using bioinformatic approaches has demonstrated that zebrafish orthologues of human genes can usually be found in chromosomal locations in the fish that reflect their location on conserved human chromosomal regions (3). In many cases, divergence in promoter sequences leads to tissue-specific activities of different teleost orthologues of a single mammalian gene.

Forward Genetics, Reverse Genetics, and Transgenesis

Simple model organisms with their rapid development and large numbers lend themselves well to the identification of new oncogenes, tumor suppressors, and novel therapeutic targets important in cancer. This is generally accomplished using classical (and variations on classical) genetic screens. Forward genetic screens are based on Mendelian inheritance of genes, and the observation of a phenotype in cells or organisms where a gene has been disrupted (Figure 8-3). The methods by which genes are disrupted and the assays used to screen for phenotypes are wide-ranging and some are species-specific. Early screens looked for naturally occurring phenotypes of simple processes (such as inability of yeast to grow at a certain temperature) as a method of assaying genes involved in cell division. Methods of inducing mutations in genes chemically, with retroviruses, or with transposons (“jumping genes”) have been used, often involving sufficient numbers of individuals to ensure that every gene in the genome of a given organism has been mutated at least once—known as saturation of the genome. Forward genetic approaches allow unbiased isolation of phenotypically mutant organisms, after which the gene causing the mutant phenotype can be identified. The forward genetic screen remains the most powerful strength of model organisms as a cancer model, in particular for the discovery of novel tumor suppressors. Tumor suppressors are genes whose normal functions are to regulate uncontrolled or abnormal growth or the growth of cells whose genetic integrity has been compromised. Since the cancer-causing effects of these genes can only be fully recognized when

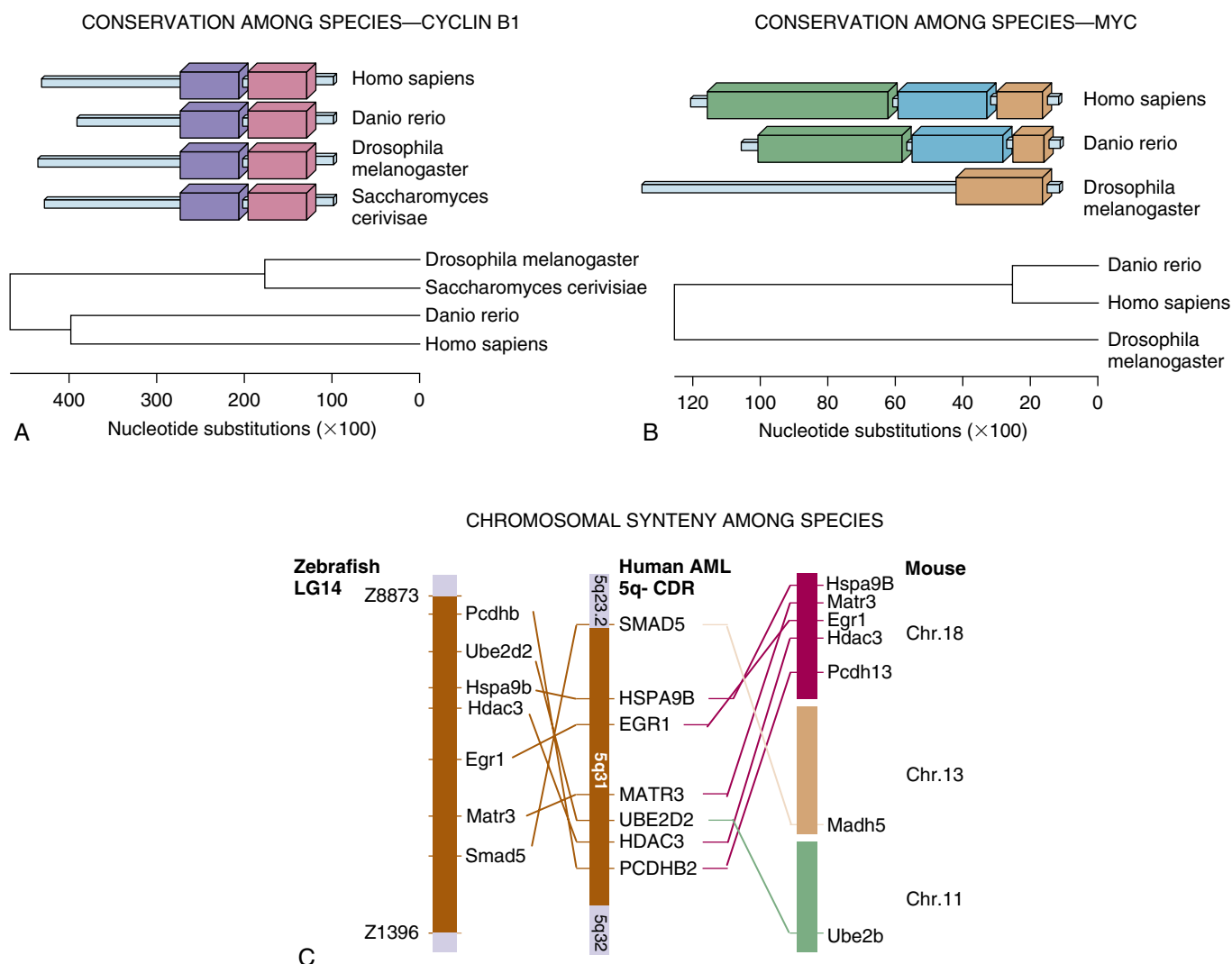


FIGURE 8-2 Conservation between species for (A) cyclin B1 and (B) the Myc oncogene. Each colored block represents a conserved protein domain. The phylogenetic trees below each block demonstrate common ancestries and the approximate number of nucleotide substitutions that have occurred leading to the divergence of the proteins for each of the given species. C: The syntenic relationships between zebrafish LG14; mouse chromosomes 18, 13, and 11; and the region on the long arm of human chromosome 5, which is found to be critically deleted in acute myeloid leukemia. AML, acute myeloid leukemia; CDR, critically deleted region; LG, locus group. (Modified from Liu TX, Zhou Y, Kanki JP, et al. Evolutionary conservation of zebrafish linkage group 14 with frequently deleted regions of human chromosome 5 in myeloid malignancies. *Proc Natl Acad Sci USA* 2002;99:6136–6141, with permission.)

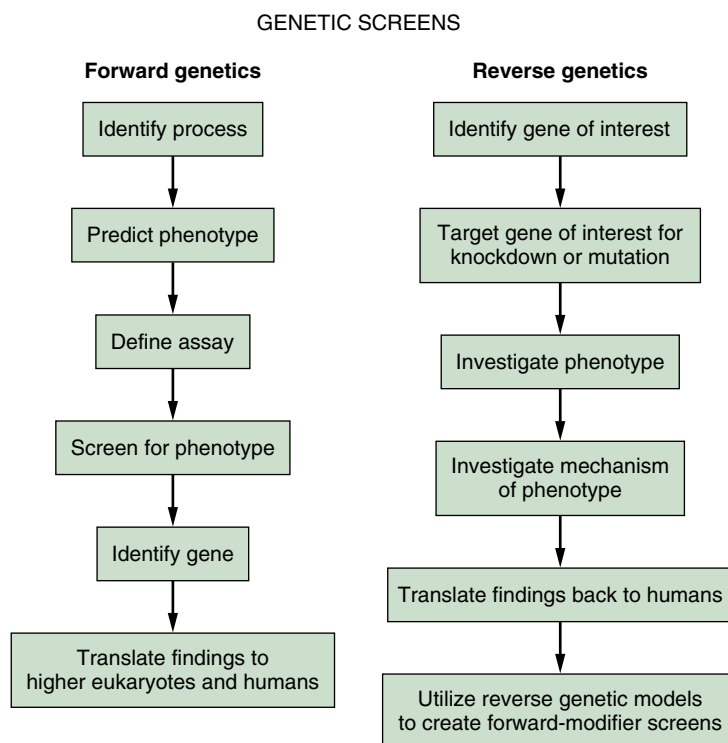
they are absent or nonfunctional, forward screening of large numbers of simple organisms is an attractive means to identify such genes *in vivo*. The advent of short-interfering RNAs (siRNAs) as a method of gene silencing has facilitated the use of cell culture systems for investigation of loss-of–gene function phenotypes, but there is no substitute for the integrated *in vivo* systems of simple model organisms.

In addition to forward genetic modeling, it is also possible to disrupt specific known genes (such as known oncogenes or tumor suppressors) to investigate the phenotype produced. This process is termed “reverse genetics.” Forward and reverse genetic models may be used in combination. Reverse genetics in simple organisms may add tools for the investigation of the genetic interactions of a known gene by permitting the development of modifier screens where the mutant is subjected to forward genetic screening to identify genes that enhance or suppress

its phenotype. In this way, modifier screens may provide novel therapeutic targets in human cancers.

Another genetic tool used in simple model organisms is transgenesis. To obtain a transgenic organism, specific DNA sequences are typically introduced into the genome of an organism and expressed under the control of a specific promoter sequence to guide the cellular, temporal, and spatial localization of transgene expression. For example, in zebrafish, the introduction of the mouse *Myc* gene under the control of the *rag2* lymphocyte-specific promoter leads to expression of *Myc* in the thymus and the subsequent development of T-cell leukemia/lymphoma (4,5). Adding the coding sequence of green fluorescent protein to the integrated construct has the additional advantage of permitting spatiotemporal visualization of the development of cancer in this system. Transgenic approaches are easily performed in yeasts, which undergo efficient homologous recombination into their

FIGURE 8-3 Flow diagram illustrating the steps involved in forward and reverse genetic screens.



chromosomes, allowing site-specific integration of the transgene. Using this tool in yeasts facilitates reverse genetics by replacing the normal functioning gene with a mutated or nonfunctional form (which may be genetically engineered or even derived from different species).

Drug Screens

Simple organisms can also be used for drug discovery in a variety of ways. Yeast can be manipulated to express a gene or protein of interest either at a higher level than normal or a heterologous human gene under control of a yeast-specific promoter. A differential effect of a drug on the normal-versus-mutated gene can also be assessed (for example attempting to find drug targets selective for certain oncogenes; 6).

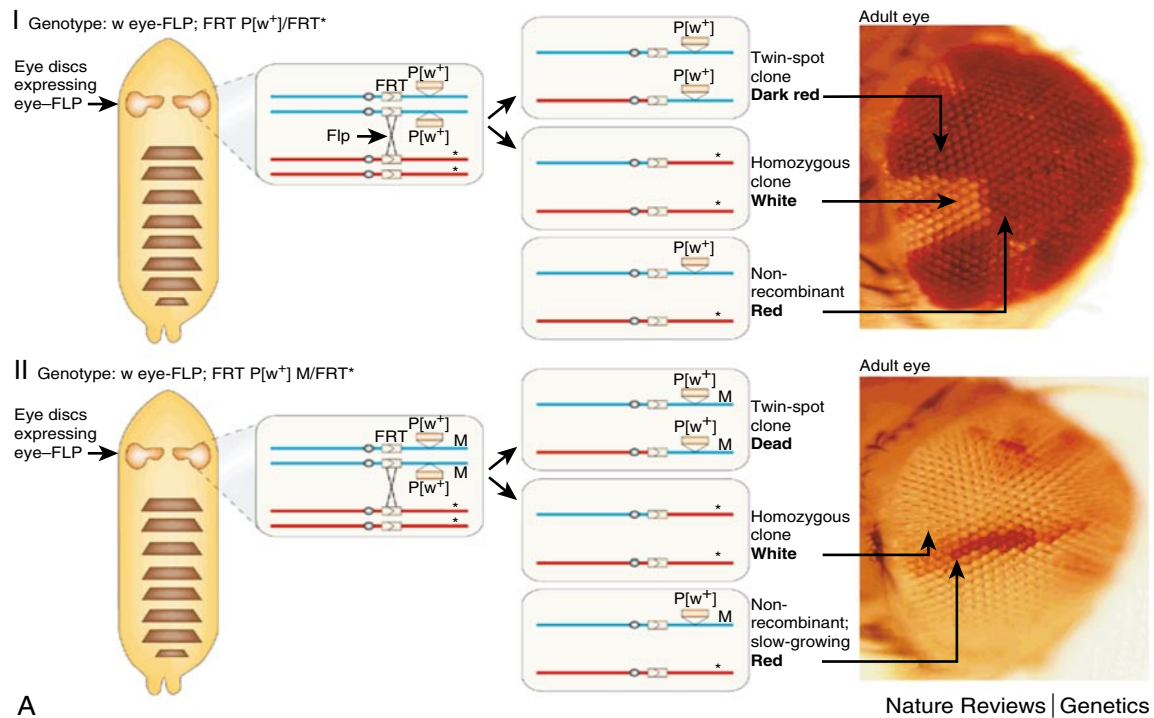
Conditional Models

A small number of syndromes predisposing to cancer in humans such as Fanconi anemia, Li-Fraumeni syndrome, Bloom syndrome, and ataxia-telangiectasia are known to be associated with specific underlying genetic mutations. Cancer in such individuals arises as a result of additional tissue-specific somatic mutations that give cancer cells their final growth advantage required for the fully transformed phenotype. In most human cancers, no predisposing gene mutations have been identified, and a series of somatic mutations are required for cancer to develop. This knowledge highlights two critical issues. The first is that the presence of one cancer-predisposing mutation can facilitate the development of other cancer-causing

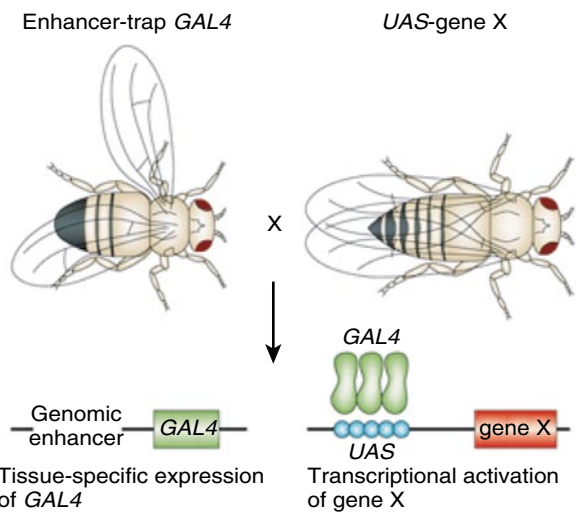
mutations—this was first postulated as the “mutator hypothesis” by Nowell in 1976 (7). The second is that the local environment in which the first and/or additional mutations occur may play a role in cancer development. Because many human oncogenes and tumor suppressors play a critical role in embryologic development, germ-line mutations affecting key genes of this type in all tissues frequently lead to death during development. While investigation of the embryologic phenotype in model organisms continues to provide crucial information into gene function, the study of genetic interactions in specific tissues and in specific cancer models provides additional information into how the mutated gene contributes to tumorigenesis in vivo.

Over the last 20 years genetic tools have been developed to engineer targeted conditional expression or (in some cases) knock-out of a specific gene. There are three main conditional systems that facilitate directing gene expression to a specific cell type at a specific time.

P1 is a bacteriophage that infects the bacterium *Escherichia coli*. This virus produces an enzyme called Cre recombinase that cuts DNA whenever it sees two identical 34-bp sequences known as Lox-P sites. The enzyme removes the DNA between the two Lox-P sites and the sites are ligated together. In model organisms, this system can be used by driving the expression of Cre with a promoter that is only expressed at a certain site (tissue specific) or with a promoter that is activated by exposure to a specific stimulus such as heat shock or a particular drug (e.g., estrogen). Lox-P sites can be introduced into a transgene such that on Cre activation, the transgene is expressed (or removed) in a specific tissue depending on how the construct was made (and in which organism). Fluorescent proteins can also be incorporated into transgenes to allow visualization of where the transgene is expressed and where the Lox-P sites have been removed (Figure 8-4). In a similar



A



B

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FIGURE 8-4 See Legend on next page.

FIGURE 8-4—Continued **A:** Flt/FRP system/twin-spot clones. By placing the FLP recombinase gene under the control of the eyeless enhancer (which drives expression specifically in the eye-antennal imaginal disc), FLP/FRT-mediated recombination can be targeted to this disc to generate homozygous mutant clones in the eye in flies that are otherwise heterozygous. The nonmutant chromosome (asterisk indicates mutation) is marked by a miniwhite transgene, but there is no selection against the twin-spot clones or nonrecombinant cells, and both the mutant clones (white) and the twin-spot clones (darker red, because they carry two copies of white+) are relatively small. The effects of incorporating a minute mutation (M) onto the nonmutant FRT chromosome. The mutant clones now occupy almost all of the eye, because they outcompete the slow-growing nonrecombinant cells (which are M/+), whereas the twin-spot clones die. **B:** Gal4/UAS system. The yeast transcriptional activator Gal4 can be used to regulate gene expression in *Drosophila* by inserting the upstream activating sequence (UAS) to which it binds next to a gene of interest (gene X). The GAL4 gene has been inserted at random positions in the *Drosophila* genome to generate “enhancer-trap” lines that express GAL4 under the control of nearby genomic enhancers, and there is now a large collection of lines that express GAL4 in a variety of cell types and tissue-specific patterns. Expression of gene X can be driven in any of these patterns by crossing the appropriate GAL4 enhancer-trap line to flies that carry the UAS-gene X transgene. This system has been adapted to carry out genetic screens for genes that give phenotypes when misexpressed in a particular tissue (modular misexpression screens). **C, D:** Cre-Lox system. Zebrafish carrying the construct shown in **(D)**. (i) No cre activation, fish show red thymus, showing that the dsRED cassette has not been excised. (ii) Following cre injection the dsred stop cassette is removed allowing expression of the myc-EGFP fusion oncogene. This fish has an enlarged green thymus indicating the development of T-cell acute lymphoblastic lymphoma. (iii) In this older fish the whole fish fluoresces green indicating disseminated T-cell acute lymphoblastic leukemia. **A and B** reproduced with permission of the Company of Biologists. Figure **C** courtesy of Hui Feng (unpublished data).

fashion, FLP recombinase is an enzyme made by the 2- μ m plasmid of *S. cerevisiae*. This recombinase acts in a similar way to Cre recombinase, recognizing two 34-bp sequences called Frt sites. This system has been extensively used in flies, where instead of simply excising the intervening DNA sequence between two Frt sites, FLP recombinase results in the crossing over and exchange of genetic material between arms when Frt sites are located on opposite arms of the same chromosome. This allows for example, expression of mutant tissue in an otherwise wild-type background (Figure 8-4; 8). Tissue-specific overexpression of a gene can also be achieved by using the yeast transcription factor Gal4 driven by a tissue-specific promoter and its upstream activating sequence (UAS) driving the gene of interest. UAS can also drive a fluorescent protein-colored marker to allow spatial localization of cells expressing the gene of interest (Figure 8-4).

Yeast

The Cell Cycle

The fundamental processes of cell growth and division are governed by the tightly regulated processes that maintain the cell cycle. The cell cycle is an ordered set of events by which cells grow and divide to produce two identical daughter cells. It is divided into four phases as shown in Figure 8-5. Two features of cancer cells in mammalian systems are controlled (at least in part) by cell cycle maintenance, altered growth, and genomic instability. Based on a number of features, yeasts provide the ideal system for study of the cell cycle (9). The budding yeast, *S. cerevisiae* has been the most commonly investigated. *S. cerevisiae* is able to survive in haploid and diploid states. In the presence of sufficient cell nutrients, diploid cells undergo division by mitosis and growth by budding. The size (or presence) of a bud can be visualized by light microscopy and permits determination of cell cycle status in individual cells. Each diploid cell contains a copy of each of the two mating types—*Mata* and *Mata* determined by the MAT locus. When diploid cells are starved of nitrogen and fermentable carbon they undergo sporulation and commence formation of *a* and *a* gametes. Here the cells undergo division by meiosis followed by differentiation of the subsequent haploid progeny. The haploid progeny immediately fuse with a cell of the opposite mating type to produce a diploid cell—a process determined by a mating pheromone that is specific to each mating type (*a* or *a*). The mating pheromone of the budding yeast is responsible for maintaining the cells in a nondividing state in

order to allow mating to proceed and thus is a negative growth regulator. These observations led to the hypothesis that mutant yeast cells unable to undergo sexual conjugation or those that have arrested at different parts of the cell cycle (usually under specific conditions such as high temperature) have disrupted genes critical to progression through the cell cycle and cell growth, which may be potential oncogenes and/or tumor suppressors (10,11). The study of mutant yeast strains with abnormalities in the cell cycle has led to the identification of genes orthologous to human oncogenes and tumor suppressors.

Genetic Tools and Functional Genomics

Recovery of the genes responsible for a mutant phenotype observed in a forward genetic screen and harnessing the power of reverse genetics is made possible in yeast by the ease with which the organism can be transformed with DNA using plasmid vectors and by the subsequent ability of the yeast to undergo the process of homologous recombination. Homologous recombination is a conserved DNA maintenance process that allows recognition of homologous DNA at meiosis and crossing over of genetic material between homologous segments. This physiologic mechanism is used when cloned DNA is incorporated into the yeast genome at its appropriate location using the integral yeast machinery. In this way, any gene can be replaced by another stretch of DNA. This could be a mutated or null allele or a normal allele to replace a mutated one. In addition, mutant or wild-type alleles can be coupled with genes encoding positive or negative selection factors to allow the identification of yeast strains that have undergone homologous recombination. The application of these techniques is widespread, including the development of targeted gene “knock-outs” such as those used in the yeast genome deletion project. An example of this is shown in Figure 8-5.

Because the yeast genome is relatively small and contains little redundant DNA, plasmid libraries have been made of all the genes found in yeast. This is done by cutting the whole genome with restriction enzymes that recognize a particular DNA sequence and then inserting each of these smaller cut pieces of DNA into a plasmid vector. These DNA libraries are useful to identify the gene causing a phenotype or to address the phenotype resulting from the overexpression of certain genes (which can also be used in a drug screen for example).

Yeasts screens have been devised to determine not just genetic but also protein–protein interactions. These are termed “two-hybrid screens.” The principle of the screens is that transcription

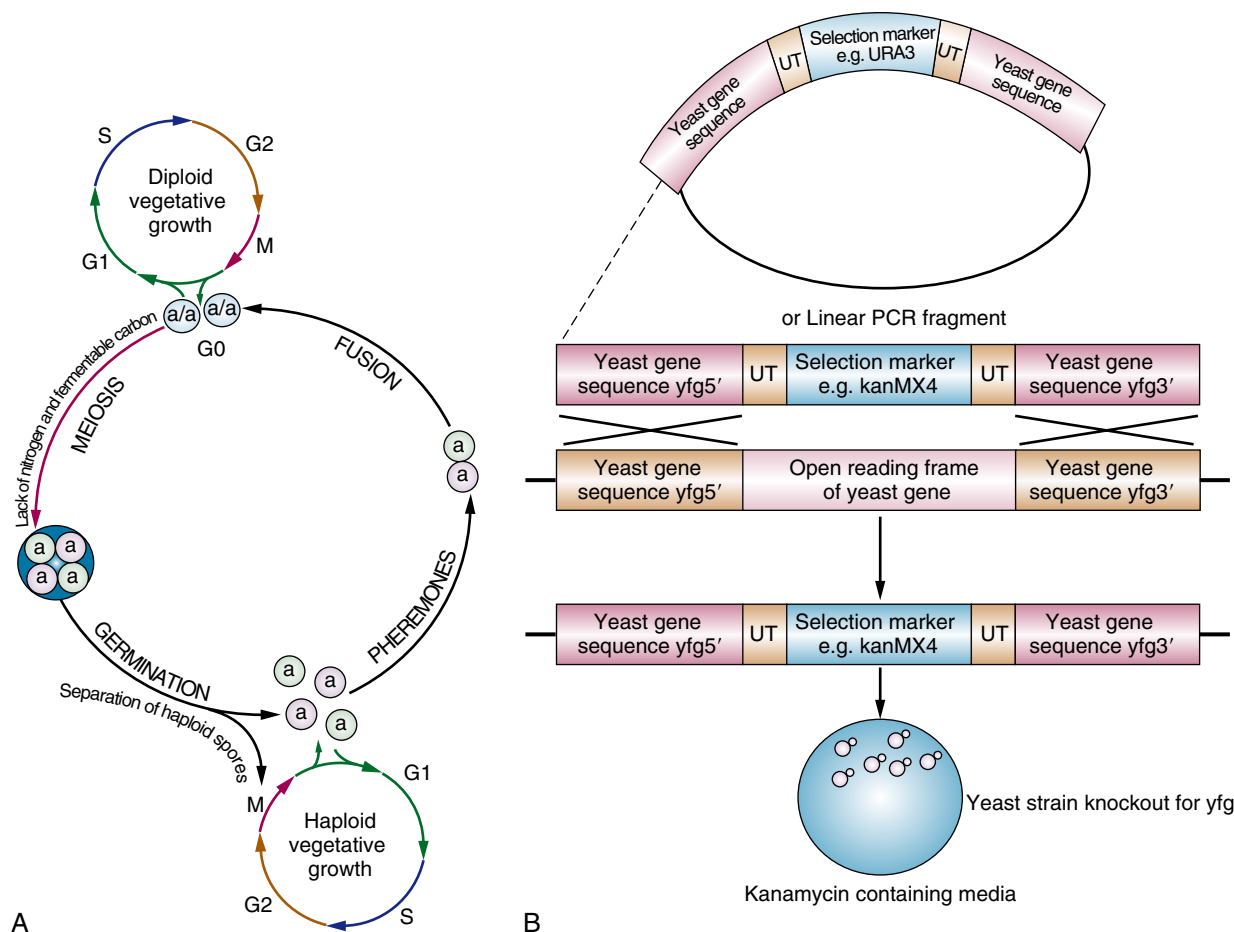


FIGURE 8-5 **A:** A schematic representation of the budding yeast life cycle. Under adequate nutritional conditions diploid yeast cells undergo vegetative growth by mitosis (**top left**). The cells cycle through G₁, S (synthesis), G₂, and M (mitosis) phases. When starved of nitrogen and fermentable carbon, sporulation occurs with formation of gametes by meiosis. The gametes are contained within a casing called an ascus. As the gametes germinate, the haploid spores separate. The **a** and **a** gametes secrete pheromones leading to fusion of an **a** to **a** gamete when they meet. If separated, however, haploid vegetative growth can also continue by mitosis (**bottom right**). **B:** Schematic representation of replacement of yeast gene *yfg* (your favorite gene) with a deletion cassette containing the selection marker *URA3* (orotidine-5'-phosphate decarboxylase). The 40- to 50-bp of *yfg* gene sequence at the 5' and 3' ends are cloned with *URA3* selection marker between. The *yfg* sequences are recognized by the homologous recombination machinery in the yeast, and a proportion of yeasts will swap the genetic material encoding the gene for that encoding the selection marker. *URA3* causes resistance to 5-FOA (5-fluoro-orotic acid); thus, yeasts that survive in media containing 5-FOA have incorporated the deletion cassette and no longer contain a functional *yfg*. (From Kumar A, Snyder M. Emerging technologies in yeast genomics. *Nature Genet* 2001;2:302–312, with permission.)

factors require two domains: a DNA binding domain (BD) and an activation domain (AD) in close proximity to one another to bind to and cause transcription of its target gene. Part of the Gal4 transcription factor (either the BD or the AD, for this example the BD) is fused to the protein of interest, for example the MYC oncoprotein. A library of other proteins is then fused to the Gal4 AD. If any of the library proteins interact with MYC then the Gal4 transcription factor will bind and cause transcription of target genes. The target gene can be engineered to be a selection factor that will only allow yeast to grow in the presence of its expression in selective culture conditions, and its identity can be confirmed by subsequent sequencing (12).

Cdc2 and Cdc28

In 2001, the power of yeast as a genetic tool for studying cell cycle and its implications in cancer were recognized with the award of the Nobel Prize for medicine to Paul Nurse and Leland Hartwell. They not only elucidated many of the critical genes involved in regulating the cell cycle, but also demonstrated the genetic and biochemical

conservation that makes study in this and other simple organisms so powerful. Two of the critical genes identified by these investigators continue today to provide insight into how cancer cells evade normal growth and cell cycle regulatory mechanisms. The *cdc28* gene was discovered in the early 1970s by Leland Hartwell in an early genetic screen to identify *S. cerevisiae* strains with abnormalities of the cell division cycle (*cdc*) that were present at high temperatures. The presence of *cdc28* was found to be essential for cells to initiate both the nuclear and cytoplasmic events required for cell division. Prior to the *cdc28*-dependent step, yeast cells are able to undergo sexual replication or entry into the cell cycle, and thus the *cdc28* step in G₁ came to be known as the start of the cell cycle in the budding yeast. The *cdc28* gene was cloned in 1980, and by 1985, *cdc28* was shown to have protein kinase activity (13).

Paul Nurse uncovered a critical regulator of the cell cycle in *S. pombe*, using a similar search for temperature-dependent mutants, and named the regulator *cdc2*. In 1982, it was shown that the *cdc28* gene in budding yeast and the *cdc2* gene in fission yeast were functionally homologous and the cloning of the human *CDC2* gene in 1987 confirmed the utility of this system for investigating the role

of specific genes in humans (9). In more recent years, *cdc2* and *cdc28* have been renamed *cdk1* (cyclin-dependent kinase-1) as part of the *cdk* family of kinases, which (along with other roles in cell cycle, transcription, and differentiation) associate with cyclins allowing intricate control of cell cycle. CDK1 associates in particular with cyclin B and is involved in the control of mitosis (reviewed in [14,15]). Subsequently abnormal expression of CDK1 has been found in a variety of human cancers and is required for efficient phosphorylation of the Bloom syndrome DNA helicase (BLM; 16). Homozygous mutation of the BLM leads to Bloom syndrome—a condition in humans that predisposes to multiple forms of cancer as a result of genomic instability.

Ploidy, Genome Instability, and Cancer

The study of Leland Hartwell's temperature-sensitive cell division mutants led to the analysis of genes essential for cell cycle progression and their roles in determining the fidelity of genetic replication. Several cell cycle mutants demonstrated a marked increase in the rates of chromosome loss, recombination, or mutation, and so the DNA damage checkpoint and DNA repair during the cell cycle were first investigated. The question has been addressed of whether polyploidy, (increased numbers of chromosomes), might cause specific genetic phenotypes, such as lethality, in a mutated gene that demonstrates no phenotype in its haploid or diploid mutated state (17,18). It is known that polyploidy increases genomic instability and occurs frequently in human cancer cells. Polyploidy has also occurred frequently throughout evolution, demonstrable by the preserved gene orientation and order resulting from ancient genomic duplications seen in yeasts and in higher vertebrate organisms. To address the potential role of mutated genes in a triploid or tetraploid state, a genome-wide analysis of polyploidy in *S. cerevisiae* has been carried out. Using a strategy of deleting a single copy of the *MATa* or *MATa* locus in diploid yeast cells also harboring a known homozygous gene mutation, viable mating diploid mutants were created (17). By mating these diploids, a genetic screen for ploidy-associated lethality has identified 17 genes falling into three functional groups—genes required for repair of DNA damage by homologous recombination, genes required for sister chromatid cohesion establishment and dissolution, and genes required for normal function of the mitotic spindle. This work demonstrated for the first time the increased requirement of polyploid cells for homologous recombination, explained by increased amounts of spontaneous DNA damage associated with the replication of an extra set of chromosomes. There is some evidence that human cancer cells with increased ploidy also require increased recombinatorial repair and elevated amounts of recombination proteins, demonstrating that genes and genetic programs identified in yeast continue to assist not only in elucidating the physiologic mechanisms behind cancer phenotypes but also potentially identifying novel future drug targets.

Flies

Why Use a Fly?

D. melanogaster has been used as a model organism in developmental biology and genetics for a century, and as such, the

generation of a vast array of tools makes it one of the most comprehensively genetically tractable systems for study. Several features of the fruit fly have led to its popularity as a genetic model, most critically the small number of genes contained within the four fly chromosomes, relatively little redundant DNA, and large numbers of human homologues that have been identified following the complete sequencing of both the fly and the human genomes. In addition, fruit flies develop large polytene chromosomes in the salivary gland. These chromosomes are produced in the last larval stage (the third larval instar) when large amounts of glue proteins are required for pupation. The large amount of protein production is achieved by genome amplification by a process called endoduplication: DNA replication without division. When stained by standard G-banding, the resolution of endoduplicated chromosomes is an order of magnitude greater than that seen of human chromosomes, as there are multiple copies of each gene. This in turn facilitates the identification of genes that have been deleted. Although wild-type flies do develop tumors, the similarities of these spontaneous growths to mammalian cancers are limited. However, forward genetic analysis has uncovered cancerlike proliferations in the developing fly larva that provide an excellent platform for the investigation of tumorigenesis in this multicellular organism. Early in embryogenesis, cell fate is assigned, leading to formation of certain adult structures, which develop in the larva via saclike invaginations of specialized epithelium (known as imaginal discs). There are 15 imaginal discs, seven bilaterally symmetrical pairs, and one germ cell imaginal disc. Imaginal discs consist of a single layer of cells, which can be easily visualized in developing larva. In addition, both brain and blood cell neoplasia can be seen in mutant fruit flies (19). Not all aspects of human cancer can be modeled in a fly, however. In particular, flies lack a closed vascular system and thus angiogenic properties of tumors cannot be investigated. Despite this, genes known to regulate the angiogenic properties of human tumors, such as vascular endothelial growth factor (VEGF), have fly homologues that have been implicated in tumor development in flies.

Another feature of the fruit fly that makes it an attractive model to study is the availability of large banks of mutant flies. Mutagenesis in flies has been performed using x-rays, chemical agents to induce point mutations, and P-element mediated insertional mutagenesis. P-elements are transposons or sequences of DNA that can move around or “jump” within the genome. When transposons insert into the genome at the beginning of a coding sequence, that gene's transcription is disrupted, generally creating a null allele. Identification of the disrupted gene is much easier than with a chemically induced point mutation, because the sequence of the P-element is known and can be “tagged” and polymerase chain reaction (PCR) primers used to facilitate gene identification. To determine which flies carry the P-element, its sequence can also be modified to carry a marker, such as *rosy* eyes. “Jumping” of the P-element requires the function of another gene, transposase, and thus the disrupted gene will be fixed unless the transposase is present. The transposase gene can be bred into flies carrying a P-element to induce the latter to move and is usually carried on what is known as a “balancer” chromosome with another mutation that is easily identifiable, such as curly wings.

Genetic Tools

The first tumor suppressors identified by forward genetics in flies exhibited only one or two features of cancer. The phenotypes observed were of hyperplasia or neoplasia in a single tissue affecting 100% of flies with the mutations and, unlike in human cancers, there did not appear to be a requirement for the development of additional mutations to cause tumorigenesis. Because these initial tumor suppressor genes were not homologous to those found in humans, this led to a decline in the popularity of the fly as a model organism to study cancer. However, this soon changed, as highly conserved signaling pathways were subsequently identified in flies and humans and more sophisticated genetic techniques to study gene interactions became available (20), including genetic screens to identify second site modifiers of known tumor suppressors include the discovery of *Drosophila* homolog of C terminal src, *dcsk*, isolated by Stewart et al. (21) in a screen for dominant modifiers of the *lats* tumor suppressor and the dissection of a number of second-site modifiers of the transcription factor E2F (22,23).

More recently, the use of the conditional Frt/Flp and Gal4/UAS systems has been invaluable in cancer research in the fly. Focusing on the fly's greatest strength—the ability to investigate cell–cell interaction at a single cell level—the introduction of mosaic clone analysis for the first time underpinned the role of the microenvironment and non–cell autonomous cues in the life of an individual cell (24). The utility of this tool has been used to dissect the cell autonomous and non–cell autonomous roles of the *Drosophila myc* gene (25–27). Similarly the interactions between oncogenes and tumor suppressors have been evaluated in mosaic clones demonstrating cooperation between many known oncogenes and fly tumor suppressors (28–31).

The normal processes by which cells migrate during different developmental processes have been studied extensively in the fruit fly. Different processes use different modes of migration, requiring alterations in cell polarity, cell shape, and the adhesion of cells to both other cells and to the extracellular matrix. Disruption of genes and signaling pathways used in normal migration processes have been shown to be involved in the ability of cancers to invade local tissues and metastasize to distant regions. This area of research is in its infancy, but the extensively delineated normal processes will undoubtedly assist in the investigation of mechanisms by which cancer cells evade their local environment in this model (8).

Numerous ingenious second site modifier and overexpression screens have been developed in *Drosophila*, the complexity of which are beyond the scope of this chapter but are extensively reviewed elsewhere (1,8,32).

Malignant Neoplastic Tumor Suppressors in *Drosophila*

Many fly mutant genes were classified as tumor suppressors in early embryonic screens for larval tissue overproliferation. However subsequent characterization of a number of those genes demonstrated mechanisms of tissue expansion that did not represent features normally ascribed to cancer cells, and thus these genes are no longer considered true tumor suppressors. However, the

recent identification of the tumor suppressor *scribble* was through a screen designed to identify maternal effect mutations that disrupted aspects of normal epithelial morphology (28). It was noted that *scribble* mutants, in addition to disrupting epithelial morphology in the embryo, also led to epithelial defects in the monolayer epithelium of the female germ cells (follicle cells) in which clones of mutant cells were expressed among wild-type cells (28). A further screen for additional mutant clones affecting the follicle cell epithelium led to the identification of another mutant with an almost indistinguishable phenotype to the *scribble* mutants. Mapping of this mutation revealed it to be an allele of a previously identified tumor suppressor called *lethal giant larvae* (*lgl*). It was also noted that mutant clones of another known tumor suppressor, *discs large* (*dlg*), led again to a very similar phenotype in follicle cells. In normal tissues, the role of the Scribble protein is in maintenance of cell polarity and cell–cell adhesion, by controlling the localization of other proteins within epithelial cells in order to maintain correct spatial orientation. Bilder et al. postulated that given such similarities in phenotype that *scribble* may be a tumor suppressor like *lgl* and *dlg*, and also that *lgl*, *dlg*, and *scribble* might interact to form the necessary machinery to maintain cell architecture and cell proliferation in fly epithelium (28). The identity of *scribble* as a tumor suppressor was confirmed by investigation of the epithelium of the third instar larvae imaginal discs, which demonstrated cellular overproliferation with loss of apicobasal polarity and disordered architecture. In addition, overgrowth of brain tissue was observed in *scribble* mutants—another feature common to the *lgl* and *dlg* mutants. Epistatic relationships between the three genes have also been demonstrated by the ability to enhance the phenotype of *scribble* mutants by an additional heterozygous mutation of *dlg* or *lgl*. Following the discovery of *scribble* and its properties as a tumor suppressor in flies, a further screen set out to use the fly as a method of studying the metastatic properties of tumors. Until this point individual tumor suppressors and oncogenes that had been studied in flies apparently lacked the capability to proliferate without the micro-environment in which the malignant cells reside. This is perhaps not surprising given the premise that a single genetic lesion is rarely sufficient to promote tumorigenesis; rather, it creates a mutator phenotype predisposing to the additional mutations required for cancer to develop. In an organism such as the fly with such a short lifespan, the likelihood of that secondary mutation occurring is low within its natural lifespan. The screen that was developed investigated the interaction of activated *ras* (*ras*^{v12}) and known and unknown additional mutations. The system allowed development of clones of malignant cells that expressed *ras* plus additional mutations in the normal microenvironment within the eye disc, using the Frt/Flp system, the Gal4/UAS and the Gal80 suppressor to localize expression. The results demonstrated that the combination of a *ras*^{v12} mutation and *scribble* mutation led to circulating tumor cells within the fly hemolymph open circulation and the development of widespread metastatic tumor formation. In these metastatic tumors, basement membrane integrity was breached (as in mammalian metastatic tumors) and overexpression of the junctional adhesion protein E-cadherin suppressed the metastatic behavior of the tumors, which is also in keeping with mechanisms of metastasis in human epithelial tumors, where

E-cadherin is frequently down-regulated (33). These studies unequivocally demonstrate the utility of the fly as a cancer model with unique properties for uncovering novel genetic interactions and potential therapeutic targets. Studies on the human SCRIBBLE gene have shown it is down-regulated in cervical cancer (34,35) and most cases of invasive breast cancers (29) and interacts with the adenomatous polyposis coli gene, leading to altered expression in many cases of colon cancer (36).

Archipelago

The archipelago (*ago*) gene was identified in a screen to identify mutant clones in eyes of fruit flies that provided a proliferative advantage over their wild-type neighbors. The screen identified several known tumor suppressors as well as three alleles of a novel gene the authors named archipelago. The *ago* mutant clones showed increased proliferation compared with wild-type and only a small amount of compensatory apoptosis. The *ago* encodes an F-box protein. F-box proteins are involved in recognition of other proteins, such as *myc* and *cyclin E*, which are targeted for degradation by a series of enzymes that catalyze the addition and polymerization of the small protein ubiquitin. These specificity factors are termed “E3 ubiquitin ligases.” Polyubiquitination directs the protein to the proteasome for degradation. The F-box protein exists in a complex with other enzyme components required for ubiquitin activation (E1) and ubiquitin conjugation (E2). In *ago* mutants, all three alleles were found to be mutated in the domain of the protein known to be involved in substrate recognition (known as the WD repeats). This led to the hypothesis that the mutant *ago* was unable to recruit a protein substrate for degradation and this in turn was responsible for the observed phenotype. Because of the proliferative phenotype, the authors hypothesized that a positive regulator of the cell cycle may be involved in the observed phenotype and investigated expression levels of the cyclins. Levels of cyclin E protein were found to be increased without a corresponding increase in cyclin E mRNA suggesting a post-transcriptional mechanism. Cyclin E complexes with *Cdk2* (cyclin-dependent kinase-2) and degradation of this complex promotes the transition from G₁ to S phase of the cell cycle. In the presence of excess cyclin E, cells are driven to replicate their DNA prematurely, leading to genomic instability. Therefore Ago appears to be the F-box protein that directs ubiquitination and subsequent degradation of cyclin E, and the failure to degrade cyclin E is responsible for the proliferative phenotype observed. Elevated cyclin E levels are seen in a variety of human cancers including breast and ovarian cancers. The human AGO orthologue FBW7 (also known as *hAGO*, *hCDC4*, and *FBXW7*) was shown to be mutated in four cancer cell lines including 3/10 ovarian cancer cell lines and one T-cell acute lymphoblastic leukemia (T-ALL) cell line (37). A further report confirmed the role of human FBW7 and *Drosophila ago* as part of a complex of proteins responsible for E3 ubiquitination known as an SCF complex and showed reduced levels of FBW7 mRNA in breast cancer cell lines where cyclin E levels were elevated (38). Subsequent investigation has shown a small number of primary ovarian cancers have mutations in FBW7.

Fish

In 1995, Christine Nusslein-Volhard won the Nobel prize for medicine for her work on the delineation of the embryonic axes of the developing fruit fly. Notably half of her acceptance speech was dedicated to a different organism, the zebrafish (39). The major appeal of the zebrafish over other organisms as a cancer model is that it allows investigation of vertebrate tumor biology but remains amenable to embryonic and forward genetic study in a manner quite unfeasible in other vertebrates. The transparent zebrafish embryos undergo extrauterine fertilization and development. The embryos can be maintained in the haploid state and gynogenetic diploid (diploid fish derived from maternal sister chromatids—or half-tetrads) are viable to adulthood and fertile. Each female fish is capable of producing up to 200 eggs per clutch and in vitro fertilization from frozen sperm is also possible. Embryonic development is rapid, with the completion of somitogenesis in only a few days and adult fish are able to reproduce from 3 months of age onward. Although the speed of forward genetic screening is slower than in flies or yeast, zebrafish is the model of choice for large-scale forward genetics in a vertebrate organism (40,41). As a cancer model, the addition of a beating heart and closed circulation in zebrafish provides the ability to dissect additional facets of cancer biology, such as abnormal angiogenesis. In contrast to flies and yeast, fish get cancer in the wild, with macroscopic characterization and microscopic histopathology similar to those seen in other vertebrates, including humans. Exposure to carcinogens has confirmed that teleost fish are susceptible to cancer in virtually all organs and tissue types (42). Several transgenic models have been developed using tissue-specific expression of human or murine oncogenes, resulting in the development of human-like cancers. These have provided the platform for a second generation of zebrafish screens, which critically include modifier screens for genes or drugs that can affect the onset or progression of oncogene-induced tumors that are genetically based on human molecular oncogenesis. Such modifier screens provide important information for dissecting disease biology and causative pathways, as well as for the identification of new drugs and therapeutic targets. An example of such pathway interactions was demonstrated in zebrafish overexpressing the activated oncogene BRAF in fish melanocytes. These produced pigmented nevi, but when mated to a *p53* mutant line, developed fulminant malignant melanoma (43).

Gene inactivation by homologous recombination as described in yeast, flies, and mice has yet to be successfully performed in the zebrafish, but the challenge of reverse genetics has been met by several other genetic technologies. Transient gene knock-down is possible in zebrafish embryos using morpholinos. Morpholinos are chemically modified antisense oligonucleotides directed either at the translational start site of a gene, blocking protein production, or at a splice site resulting in inappropriate RNA splicing and the formation of nonfunctional proteins. Injection of the morpholino into the single-cell-stage embryos results in gene knock-down that is stable for around 4 days, allowing observation of the effects of gene inactivation on embryonic development. This allows rapid assessment of whether gene function in fish creates a phenotype similar to that in mammals.

To provide specific germ-line gene knockout models two major technologies have been used. Both are based on traditional forward genetic mutagenesis techniques. The first, Targeting Induced Local Lesions IN Genomes (TILLING) combines chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU) with an enzyme derived from celery named CELI, which cuts DNA at positions of base-pair mismatch. Using this enzyme, pools of genomic DNA from multiple mutagenized fish can be amplified by PCR to identify mutations in a gene of interest. An alternative method is large-scale viral insertional mutagenesis. This technique was pioneered in the laboratory of Dr. Nancy Hopkins and involves the use of a murine retrovirus that inserts randomly into the genome (44). A commercial company has used this technique to provide a library of fish available with viral insertions in a wide range of identified gene—the only disadvantage being that many of the insertions are intronic or in potential gene promoter regions and the ability or efficiency with which many of the insertions can knock out gene function remains undetermined.

Although in recent years the fish of choice for modeling tumorigenesis has been the zebrafish, several other teleost fish have been used for genetic study. The fully sequenced genome of the pufferfish (*Takifugu rubripes*) is particularly of benefit to zebrafish researchers, since there is less evolutionary divergence between fish genes than between those of fish and mammals, and there is less intronic DNA in the pufferfish than in the zebrafish. By comparing the gene localization and sequence in the pufferfish, the few remaining gaps in the now almost-complete zebrafish genome sequence can often be bridged.

Mouse Myc-Induced T-Cell Acute Lymphoblastic Leukemia

The role of the zebrafish as a cancer model combining the attributes of vertebrate biology and model organism genetics came to fruition with the development of T-cell acute lymphoblastic leukemia (T-ALL) in transgenic zebrafish expressing the mouse *c-myc* (*m-myc*) oncogene under the lymphoid-specific promoter *rag-2*. The *m-myc* oncogene was fused to a cDNA encoding the enhanced green fluorescent protein (EGFP) allowing real-time visualization of the leukemic cells. In common with mammalian hematological malignancies it was possible to sublethally irradiate a recipient wild-type zebrafish and transplant EGFP-positive tumor cells from the *m-myc*-induced leukemia from another fish by injecting them into the immunosuppressed recipient fish peritoneum. These fish went on to develop leukemia with the same pattern as the donor fish, with initial homing of T lymphoblasts to the thymus gland, followed by subsequent infiltration of surrounding tissues, and finally dissemination and death (5). The development of leukemia in the *m-myc*-expressing fish was so efficient that the affected fish often did not survive to reproductive age. To allow propagation of the transgenic zebrafish expressing the mouse *c-myc* (*m-myc*) oncogene under control of the lymphoid specific promoter *rag-2* without using in vitro fertilization, a conditional model was developed using Cre/Lox technology. The system allowed visualization of T-lymphocytes not expressing *m-myc* by incorporating a red fluorescent protein (dsRED2) with a stop codon flanked on

either side by lox-P sites. When excised following injection of Cre RNA at the one-cell stage, this transgene led to the development of green *m-myc*-expressing cells that went on to develop T-ALL at a median of 152 days (well after the onset of sexual maturity; 4). This seminal work has demonstrated not only the ability of fish to develop human-like cancers in response to mammalian oncogenes, but also the a feasible fish tumor model system for modifier and drug screens to alter the leukemia phenotype or onset.

Zebrafish Screen for Genomic Instability Mutants

Many inherited human syndromes predisposing to cancer (such as Fanconi anemia and Bloom syndrome) are characterized by disruption of genes critical for DNA repair and maintenance of genomic stability. Karyotypic abnormalities are a common finding in most cancers that progressively accumulate over time, highlighting a role for genomic instability in cancer progression. To identify novel genes predisposing to genomic instability and the development of cancer, a forward genetic screen in zebrafish was designed. The screen design used several unique facets of zebrafish genetics. First, wild-type male fish were treated with the mutagen ENU to induce 100 potential genomic instability (*gin*) mutations per sperm. These fish were mated to fish homozygous for a pigment mutation known as golden (*gol*). Golden embryos in the homozygous state have a characteristic gold-colored pigment in the developing eye, in contrast to wild-type or heterozygous fish, where the pigment is black. In heterozygous golden mutants, an additional recessive mutation predisposing to genomic instability will induce patches of golden pigment as second inactivating mutations occur in the remaining normal golden allele. The number and size of patches of golden tissue can be quantified. This assay is known as the mosaic eye assay (45). For this assay to effectively identify recessive *gin* mutations, fish need to be homozygous for the *gin* mutation (*gin/gin*) and heterozygous for the *gol* mutation (*gol/+*). The progeny from the initial matings is heterozygous for both (*gin/+* and *gol/+*). To obtain this configuration, early pressure parthenogenesis was used. This technique uses ultraviolet (UV)-irradiated sperm to fertilize the double heterozygous fish, leading to potentially haploid embryos. To maintain a gynogenetic diploid state, the second meiotic division is inhibited by using early pressure applied by a French press. Because of crossing over at the cell cycle stage meiosis I, genes that are nearer to the ends of the chromosomes (telomeric) compared with those nearer to the centromere (centromeric) are more likely to have undergone crossing over; therefore telomeric genes are more likely to be in the heterozygous state. The golden locus is known to be telomeric and 89% of embryos generated in this way were heterozygous for *gol*, allowing assessment of genomic instability in the mosaic eye assay. Twelve genomic instability mutants were identified in the screen, all leading to an increased incidence of a variety of cancers in the adult fish in both the heterozygous state but more markedly in the homozygous state. Additionally some of the mutations interacted with one another to produce more severe phenotypes in the double heterozygous state (46). Only preliminary mapping of the mutations has been completed, but identification of the genes causing the observed cancer-predisposing genomic instability phenotypes

will likely shed some valuable information on tumor formation in mammals including humans.

Conclusion

This chapter provides the reader an overview of the immense utility and strengths of simple model organisms as tools to dissect the molecular pathogenesis and improve the targeted therapy of human cancer. Model organisms can tell us more about which we know a little of, and reveal to us things of which we know nothing, which is especially important given the emerging complexity of genetic alteration in human cancers. Useful additional internet resources are provided in Table 8-1.

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Table 8-1 Internet Resources

<http://info.med.yale.edu/genetics/xu/flycancergenes>

http://dbb.urmc.rochester.edu/labs/sherman_f/yeast/Cont.html

http://zfin.org/cgi-bin/webdriver?Mlval=aa-ZDB_home.apg

<http://flymove.unimuenster.de/Organogenesis/ImagDiscs/OrgDiscpage.html?http&&flymove.unimuenster>

<http://www.neuro.uoregon.edu/k12/zfk12.html>

<http://www.dnaftb.org/dnaftb/>

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Genetic Mouse Models of Cancer

The study of many different organisms has contributed to our understanding of cancer at the molecular, cellular, and organismal levels. Considerable effort is focused on the rational design and use of mouse models, including spatially and temporally controlled genetic modifications to recapitulate human cancers. Long before the development of genetically engineered animal models, research on mice, rats, rabbits, and chickens led to major discoveries directly related to cancer, such as the discovery of oncogenes and the biochemical purification of tumor suppressor proteins (1–4). Additionally, many key regulators of proliferation, differentiation, and cell death have been characterized by studying developmental processes in mice. This knowledge of pathways that regulate organ development is a wonderful framework on which to build our understanding of all aspects of tumor initiation, progression, and metastasis.

In this chapter, we will discuss genetically and nongenetically engineered mouse models of cancer, emphasizing the techniques used to create genetically engineered mouse models and the application of these models to cancer research. Several fundamental discoveries resulting from the use of mouse models will also be highlighted, as well as the important role of these models in the future of cancer research.

Basis for Mouse Models of Cancer

Knowledge of the genetic alterations in human tumors and the ability to manipulate the mouse genome has allowed for the development of models of human cancer (5–8). Mice are the preferred model organism with which to study the complex processes of tumor development and progression for many reasons, including their short generation time, small size, availability of inbred mouse strains, and the close genetic relationship between mice and humans. Fish, flies, and worms have also been successfully used to investigate tumorigenesis, and the genetic tools available in these species have allowed for a range of informative experiments to be performed (9–12).

Observational and correlative studies of human cancer combined with *in vitro* experiments have contributed a great deal to the foundation of our knowledge of tumorigenesis. The dissection of cancer development and progression in humans is limited by the inability to test gene function *in vivo* except by pharmacologic means. The interrogation of gene function *in vitro* is limited to

genes that control the intrinsic processes of cancer cells including proliferation, differentiation, and cell death. Moreover, the complex interactions between different cell types within the tumor are poorly recapitulated *in vitro*, and the selective pressure of *in vitro* growth may significantly alter the genotype and phenotype of cultured cells. For these reasons, animal models of cancer that allow the entire developmental progression of the disease to occur *in vivo* are of paramount importance.

The underlying genetic heterogeneity of the human population, the existence of subtypes of different malignancies, and the genetic and genomic heterogeneity within tumors of the same type complicate studies of human tumor gene expression and mutational analysis. The induction of tumors with specific oncogenic alterations in mice on inbred backgrounds can overcome many of these limitations. Mouse models also offer the ability to assign causality to genetic alteration and to assess the roles of certain genes and pathways *in vivo*.

Mouse Models of Cancer

Modeling human cancer in mice has evolved as techniques to modify the mouse genome have been developed. Combinations of the approaches described in the following sections have been used to model many human cancers. The plethora of options to create these models has been used to address many fundamental questions in tumor biology.

Spontaneous and Mutagen-Induced Tumor Models

Mice spontaneously develop a spectrum of cancers. The observation that different inbred strains of mice develop cancer at different frequencies gave early support to the idea that the genetic background of a mouse (or person) can predispose them to cancer. Spontaneous tumor formation is often assessed in genetically engineered mouse lines to determine whether the specific gene mutation influences the prevalence, progression or type of cancers that arise (Figure 9-1A).

Many known or suspected carcinogens have been used to create mouse models of cancer (Figure 9-1B). These models rely on the treatment of mice with chemical or physical mutagens,

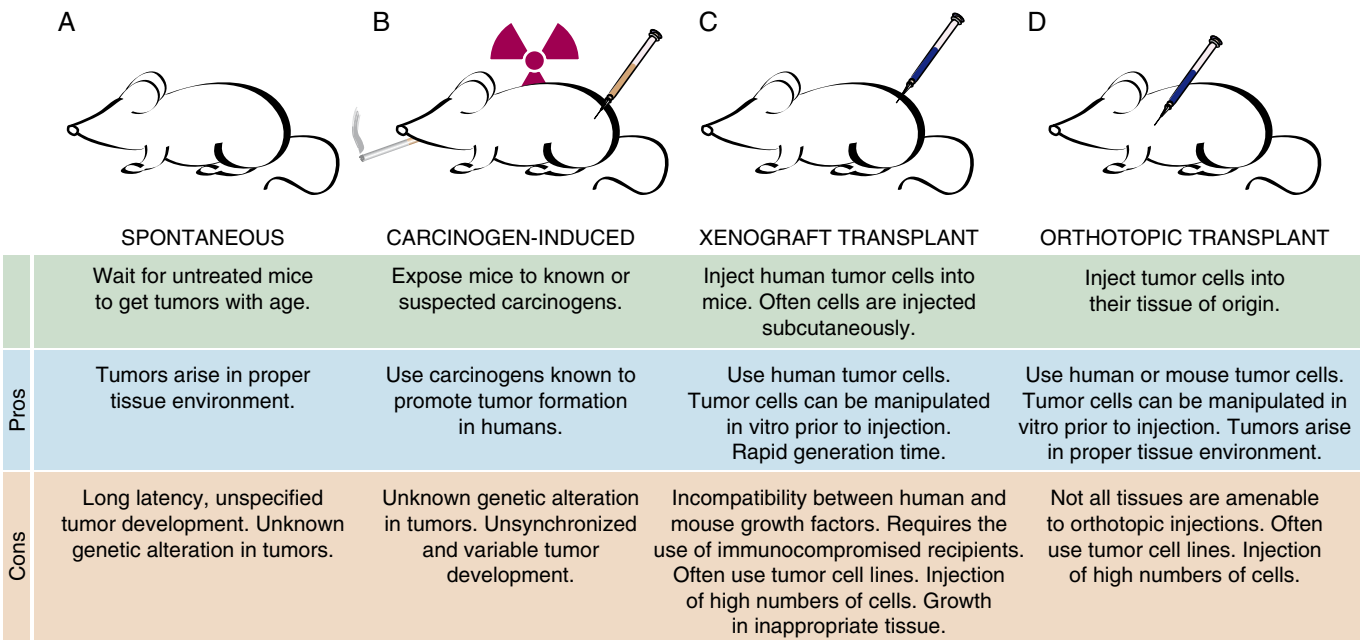


FIGURE 9-1 NONGENETICALLY ENGINEERED MOUSE MODELS OF CANCER. **A, B:** Mice develop tumors spontaneously or in response to carcinogen exposure. **C, D:** Transplantation of human or mouse tumor cells into recipient mice provides a rapid method to study cell growth and progression in vivo.

which leads to the development of largely genetically undefined cancers. Carcinogen-induced cancer protocols are most often used with genetic techniques to create combined carcinogen/genetic models of human cancer.

Xenograft and Orthotopic Models

The transplantation of human and mouse tumor cells into recipient mice has been used extensively to investigate tumor development in vivo (Figures 9-1C and 9-1D). Human tumor cell lines can be injected orthotopically into the organ from which the tumor originated, intravenously (to mimic the metastatic spread of cancer cells) or subcutaneously (to simply allow the tumor to grow in vivo) Mouse tumor cell lines can be transplanted into syngeneic immunocompetent recipients, whereas human cell lines must be transplanted into immunocompromised recipients. This in vivo tumor growth requires many of the proper tumor–host interactions, including development of vasculature and recruitment of supportive stromal cells. However, these procedures often involve the injection of high numbers of cells and do not recapitulate the series of events that lead to human cancer. Nonetheless, the ability to manipulate tumor cell lines in vitro prior to transplantation and the speed and reproducibility of tumor growth are major advantages of these approaches.

Genetically Engineered Mouse Models

Gene expression and genomic analyses of human cancers have uncovered many of the important genetic changes in different tumor types. The knowledge gleaned from these studies coupled with the ability to create germ-line and somatic alterations in the mouse genome has allowed the creation of genetically

defined mouse models of cancer that approximate human cancer at the genetic and histologic levels. Transgenic overexpression was the first genetic technology used to create mouse tumor models (Figure 9-2A; 13–15). A tissue-specific promoter can be used to drive the expression of a gene of interest in the desired cell type or tissue and the tumorigenic consequences can be determined. More elaborate transgenic approaches also allow transgene expression to be controlled temporally.

An interesting system for the delivery of genes to somatic cells in vivo uses avian retroviral vectors. Transgenic expression of the cell surface receptor for the RCAS virus (tva) allows the specific and stable infection of a cell type of interest (Figure 9-2B; 16). The viral vectors can be engineered to express a gene of interest and the effect of these genes on tumorigenesis can be determined after in vivo infection of the tva-expressing permissive cell type.

Techniques to alter the germ-line of mice allow the deletion or alteration of genomic loci (Figures 9-2C and 9-2D). These alterations can also be induced in cell type and temporally regulated fashions. These powerful approaches allow mouse models to be created that mimic the loss of tumor suppressor genes and activation of oncogenes that occur in different human cancers, resulting in mouse models that closely resemble the human disease. These genetically engineered mouse models are being used in a myriad of research settings to further our understanding of tumor biology.

Techniques to Modify the Mouse Genome

Different genetic strategies can be used to overexpress, alter, or reduce the expression of genes that affect tumor incidence or

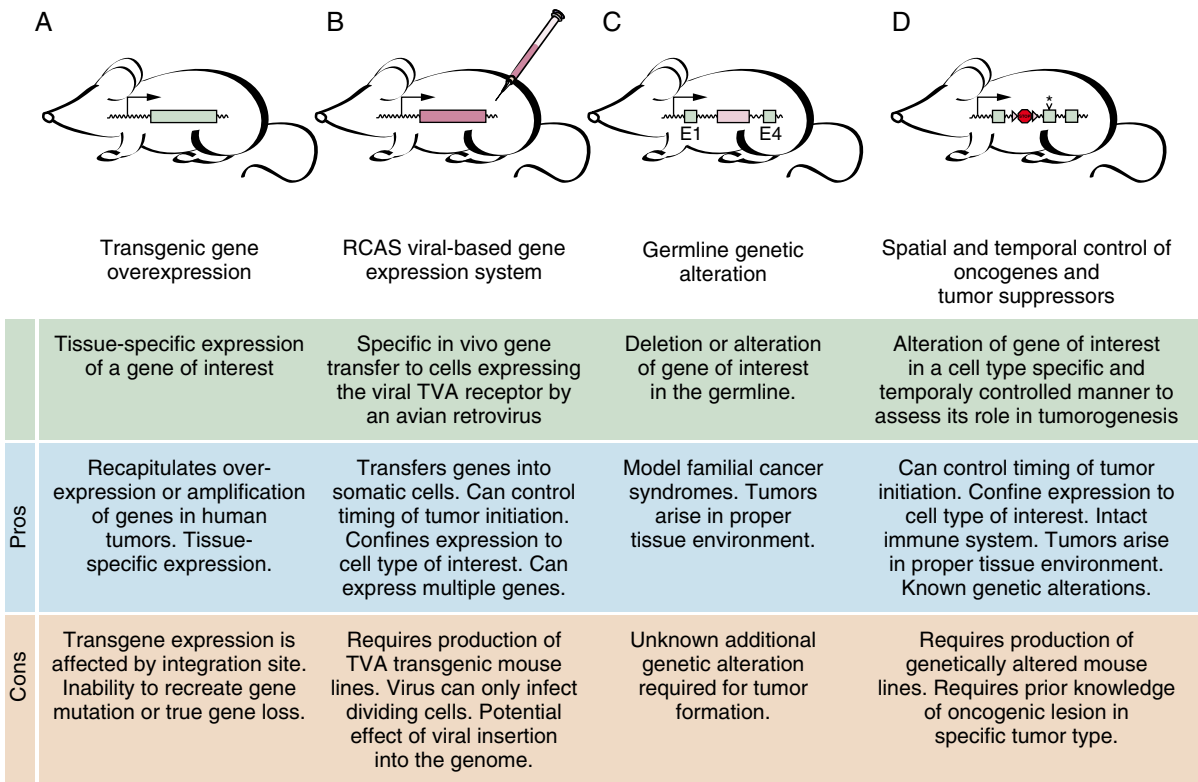


FIGURE 9-2 GENETICALLY MODIFIED MOUSE MODELS OF CANCER. **A, B:** Transgenic gene expression and **(C, D)** the alteration of endogenous loci allow induction of tumors in mice with genetic alterations analogous to those in human cancer.

progression. Genetic mouse models begin to recapitulate the selected human cancer when the genetic alterations are consistent with those detected in human cancers and when those alterations produce a tumor lesion that appears histologically similar to the human disease. Transgenic overexpression, induced and germ-line gene deletion, and conditional expression of activated oncogenes allow most of the genetic alterations found in human cancers to be genetically modeled in mice.

Transgenic Mice

Transgenic mice have an extra copy of the gene of interest controlled by a ubiquitous or tissue-specific promoter (Figure 9-3A). The use of a cell-type-specific promoter provides spatial control over the expression of the transgene. A normal or mutant form of a gene can be overexpressed to ascertain its effect on tumor development. In addition to gene overexpression, transgenic mice can also be used to reduce gene expression or protein function. The expression of dominant negative or viral proteins that interfere with endogenous protein function has been used to assess the effect of disrupting certain pathways on in vivo tumorigenesis. Additionally, RNA interference (RNAi) is an emerging technology that can be used to reduce the expression of a gene of interest in mice (Figure 9-3B).

Traditional transgenic mice constitutively express the transgene in the chosen cell type, potentially disrupting organ development or tissue homeostasis. Therefore, systems have been developed

that allow the temporal control of transgene expression or function. Two complementary systems rely on a tetracycline-dependent transactivation to control the spatial and temporal expression of the gene of interest. (Figures 9-3C and 9-3D; 17–19). The tetracycline transactivator (tTA) drives the expression of genes under the control of the bacterial tetracycline-dependent operator (tetO). The transactivation function of the tTA is blocked when tetracycline derivatives, often doxycycline, are present (Figure 9-3C). The reverse tTA (rtTA) works analogously to tTA except that the expression of the tetO-controlled gene is induced only in the presence of doxycycline (Figure 9-3D). Exposure of mice with cell type specific expression of the tTA or rtTA transgene and a tetO-controlled gene of interest to doxycycline can be used to turn gene expression on and off. These systems have allowed investigators to control tumor initiation and evaluate the requirement for continued oncogene expression during tumor maintenance and progression (20–26).

The fusion of oncogenes and tumor suppressors to hormone receptors has also been used to regulate protein function by controlling their subcellular localization (Figure 9-3E). In-frame fusion of a gene of interest to the estrogen receptor (ER) or a truncated progesterone receptor (Δ PR) creates a fusion protein that is sequestered in the cytoplasm until the cell is exposed to the appropriate hormone that induces its nuclear import (Figure 9-3E). Modified ERs (ER^{TAM} and ER^{T2}) have been created that translocate to the nucleus in the presence of 4-hydroxy-tamoxifen but not natural ER ligands, thus reducing background

Table 9-1 Examples of Genetically Modified Mouse Models of Cancer

Tumor Type	Genetic Modification	Mouse Model	Common Alterations in Human Cancer	Reference
Breast cancer (Chapter 34)	Transgenic expression of an oncogene	MMTV-HER2	<i>HER2</i> , <i>C-MYC</i> and/or <i>cyclin D1</i> amplification; germline <i>BRCA1</i> or <i>BRCA2</i> mutations; <i>p53</i> , <i>RB1</i> and/or <i>PTEN</i> loss	80, 81
Prostate cancer (Chapter 35)	Transgenic expression of the SV40 large T-antigen to block tumor suppressors	Pb-T antigen	<i>RB1</i> , <i>p53</i> , <i>PTEN</i> and/or <i>NKX3.1</i> loss; active K-RAS, active H-RAS	82
Acute lymphoblastic leukemia (Chapter 29)	Tetracycline-regulated oncogene expression (Dox off)	EμSRα-tTA; tetO-cMYC	Immunoglobulin locus-MYC translocation	22
Melanoma (Chapter 38)	Tetracycline-regulated oncogene expression (Dox on) in the absence of a tumor suppressor locus	Tyr-rtTA; tetO-Hras^{G12V}; Ink4a/Arf^{-/-}	<i>INK4a/ARF</i> loss; N-RAS activation, B-RAF activation; <i>PTEN</i> loss; <i>MITF</i> amplification, <i>NEDD9</i> amplification	24
Pancreatic B cell adenocarcinoma (Chapter 37)	Estrogen receptor–oncogene fusion regulated by tamoxifen and transgenic expression of a prosurvival gene	pIns-cMycER^{TAM}; RIP7-Bclx_L with Tamoxifen	<i>MEN1</i> loss	30
Glioblastoma (Chapter 41)	Avian virus delivered oncogene in the absence of a tumor suppressor locus	Nestin-tva; Ink4a/Arf^{-/-} with RCAS-EGFR*	<i>INK4a/ARF</i> loss; <i>EGFR</i> amplification; <i>p53</i> loss, <i>RB1</i> loss; <i>CDK4</i> amplification	16
Colon cancer (Chapter 33)	Mutation of a tumor suppressor gene	APC^{Min/+}, APC^{Δ716/+}, APC^{638N/+}	<i>APC</i> , <i>SMAD4</i> and/or <i>p53</i> loss; active K-RAS, active N-RAS	83–85
Small cell lung cancer (Chapter 32)	Deletion of two tumor suppressor genes with viral-Cre	p53^{flax/flax}; Rb^{flax/flax} with viral-Cre	<i>RB1</i> loss and <i>p53</i> loss; <i>N-Myc</i> or <i>L-MYC</i> amplification	48
Acute Myeloid Leukemia (Chapter 30)	Conditional chromosomal translocation	MLL^{loxP/+}; Enl^{loxP/+}; Lmo2^{cre/+}	Many different translocation including the <i>MLL-ENL</i> translocation	42
Pancreatic ductal adenocarcinoma (Chapter 37)	Conditional activation of an endogenous oncogene and expression of a point mutant tumor suppressor gene	Kras^{LSL-G12D/+}; p53^{LSL-R172H/+}; Pdx1-Cre	Active K-RAS; <i>p53</i> loss, <i>SMAD4</i> loss	86
Non-small cell lung cancer (Chapter 32)	Conditional activation of an endogenous oncogene and deletion of a tumor suppressor gene with viral-Cre	Kras^{LSL-G12D/+}; p53^{flax/flax} with viral-Cre	Active K-RAS; <i>p53</i> loss; <i>EGFR</i> activation and amplification	87
Head and Neck Squamous Cell Carcinoma (Chapter 40)	Progesterone-regulated conditional activation of an endogenous oncogene and deletion of a tumor suppressor gene	Kras^{LSL-G12D/+}; TGFβRII^{flax/flax}; K5-CrePR1 with RU486	<i>p53</i> loss, <i>Ink4a/Arf</i> loss, <i>cyclin D1</i> amplification, active K-RAS, active H-RAS	88

translocation (27,28). These acutely switchable protein alleles have been used to determine the execution point for various nuclear proteins, including oncogenes and tumor suppressors (29–31).

Gene-Targeted Mice

The ability to alter endogenous loci within the mouse genome has dramatically affected every field of biology (32). Homologous

recombination in embryonic stem cells allows the specific deletion or alteration of genomic loci (Figures 9-4A and 9-4B). This technique was initially used by cancer biologists to make germ-line deletions of several genes implicated in human cancer (33–36). These conventional “knock-out mice” lack the gene of interest in every cell in the animal. Germ-line deletion of some genes results in embryonic lethality, necessitating the analysis of heterozygous mutant mice or the use of conditional deletion strategies. Several

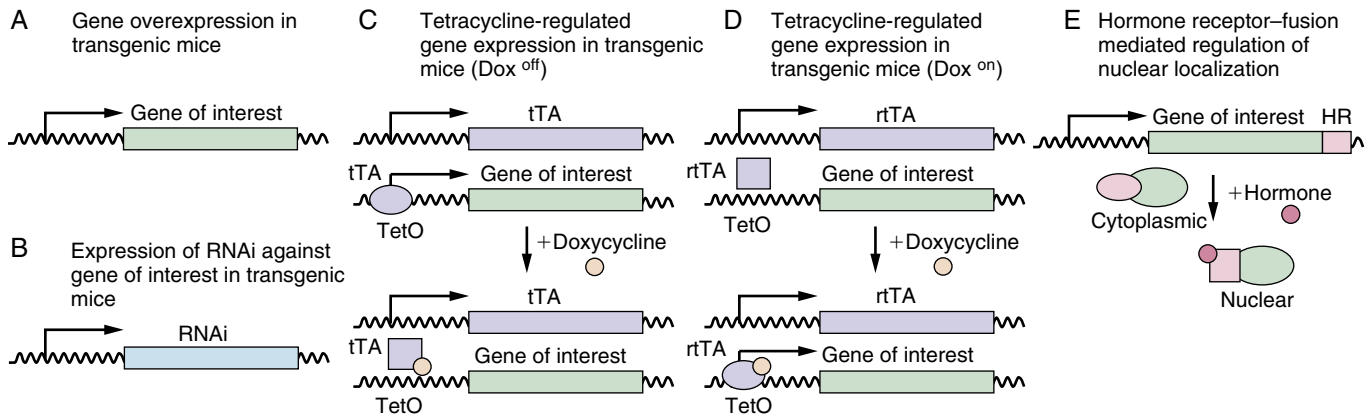


FIGURE 9-3 THE TOOLBOX FOR THE TRANSGENIC CONTROL OF GENE EXPRESSION AND PROTEIN FUNCTION IN GENETICALLY MODIFIED MICE. **A, B:** The use of tissue-specific promoters allows the expression of a gene or interfering RNA of interest in the desired tissue. **C, D:** Regulation of gene expression by the tetracycline system adds a level of temporal control based on a change in conformation of the tet transactivator (tTA) or reverse tet transactivator (rtTA) in the presence of doxycycline. **E:** The expression of hormone receptor (HR)-fusion proteins allows the nuclear translocation of proteins of interest only in the presence of the hormone.

tumor suppressor genes are mutated in the germ-lines of families predisposing them to cancer (37–40). Mice with heterozygous deletion or mutation of these genes can serve as useful models to study tumor development under these sensitizing genetic conditions (41).

The ability to delete genes specifically in a chosen cell type is comparable to the use of tissue-specific promoters to drive transgene expression (Figure 9-4C). Using bacteriophage-derived Cre recombinase, it is possible to delete genomic regions flanked by loxP nucleotide sequences (these loci are referred to as “floxed”; 32,42). FLPe recombinase is used less frequently, but can also be used to recombine loci flanked by FRT sequences (Figure 9-4C).

The development of mice that express Cre recombinase in defined cell types and the creation of floxed alleles of many genes important in cancer have allowed researchers to investigate the role of these genes in the development of various types of

tumors in a highly controlled manner. Cre recombinase can also be used to induce chromosomal translocations analogous to the translocations that are pathognomic of certain hematopoietic cancers (43,44). Through gene targeting, loxP sites can be placed at defined regions on separate chromosomes and Cre-mediated recombination between these loxP sites results in a reciprocal translocation (Figure 9-4D). The rare cells that undergo this translocation can form hematopoietic cancers similar to those detected in humans (43,44).

The expression of activated oncogenes is an important aspect of mouse models of human cancer. To express a mutated oncogene at its physiologic level from its endogenous promoter (as is the case in most human cancers), mice have been engineered with a floxed transcription/translation stop cassette in the first exon of a chosen mutant oncogene. These oncogenes remain silent until Cre-recombinase removes the stop cassette, allowing the expression of the mutant oncogene in the chosen cell type (Figure 9-4E).

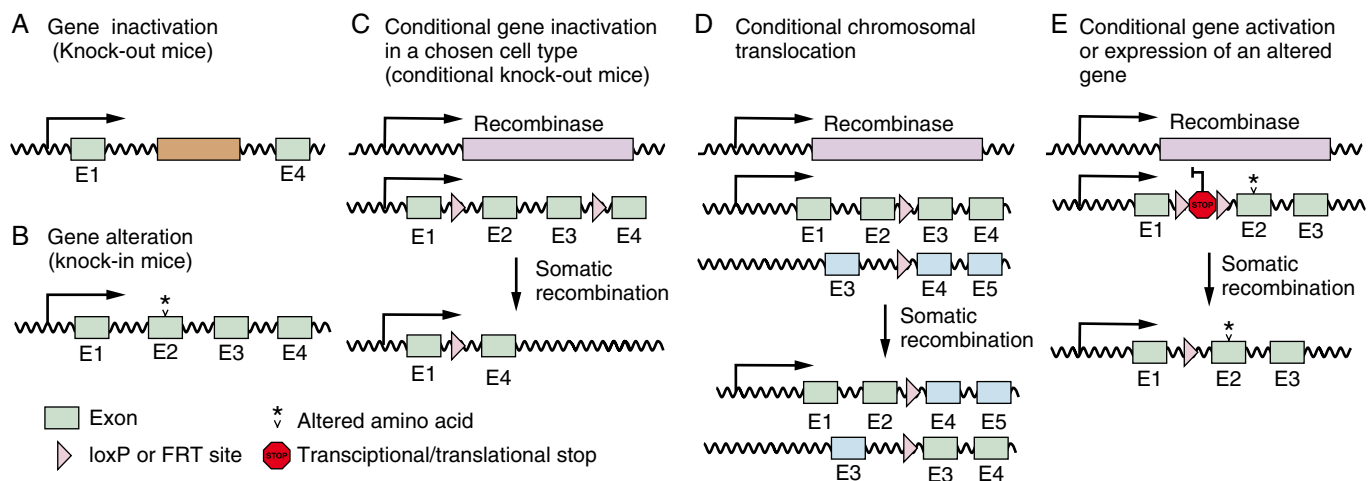


FIGURE 9-4 THE TOOLBOX FOR THE DELETION OR GENETIC MODIFICATION OF ENDOGENOUS GENES IN MICE. Genetic alteration of endogenous loci to inactivate (**A**), alter (**B**) or conditionally activate (**D**, **E**) or inactivate (**C**) genes. Homologous recombination allows the deletion or alteration of gene coding sequences. **C–E:** The expression of a recombinase (Cre or FLPe) from a tissue-specific promoter or virus allows the spatially restricted deletion (**C**), translocation (**D**), or induced expression (**E**) of a targeted allele.

Specific promoters direct the expression or deletion of genes to a specific cell lineage and sophisticated systems can also allow the timing of gene alteration to be controlled. But in these situations, every cell of the chosen cell type undergoes the same oncogenic event, which is in stark contrast to the initiation of human tumors where a single cell likely undergoes the oncogenic alteration. Although inducing these genetic changes in a single cell may not be the most appropriate approach in experimental research, the use of viruses to deliver Cre recombinase to a subset of cells may be an acceptable medium. In these systems, viruses (often Adenoviruses) are used to deliver Cre to a fraction of the cells in the organ of interest in mice that are genetically poised to express or delete genes of interest. These viral vectors have been used to initiate multifocal non-small cell and small cell lung cancer, hepatocellular carcinoma, ovarian cancer, and various brain tumors (45–48).

Rational creativity may be the underlying theme of these mouse models. Table 9-1 contains a selection of mouse models that use a variety of different genetic techniques to model different tumor types. As our knowledge of the genetic alterations in human cancers increases, our ability to control their expression in mice will also expand with the application of additional orthogonal systems.

Applications of Mouse Models to Cancer Biology

Combinations of the methods described in the preceding sections have been used to address several important questions in cancer biology, including oncogene addiction and the cooperation and interdependence of various oncogenes and tumor suppressors.

Cross-Species Comparisons

The comparison of tumors from different species has highlighted the central role for several oncogenic and tumor suppressor pathways. Mutations in *p53* are found in about half of human tumors but *p53* is also mutated in tumors in the soft-shell clam, *Mya arenaria*, underscoring the importance of this tumor suppressor and the conservation of critical alterations across diverse phyla (49,50). Cross-species comparison of gene expression and genomic changes in tumors from mice and humans has also yielded valuable insight into the important genetic changes in cancer (51–54). The genetic changes in human tumors are often complex and are overlaid on the considerable allelic variation between individuals. Although possible, pinpointing the important somatic changes or genomic alterations can become unwieldingly complex (55). By comparing the overlapping genomic and genetic changes in mouse and human tumors of the same type (and even tumors containing several of the same oncogenic events), the minimal critical genetic changes can be established. Additionally, these changes can be functionally validated in the mouse models that aided in their identification.

Oncogene Addiction

Mutation or overexpression of oncogenes can initiate tumor development. The use of tetracycline regulated expression systems has

documented that oncogene expression is also required for continued growth and survival of established tumors and metastases (22–26). Although most tumors undergo dramatic cell death and regress after oncogene inactivation, the regression is not always complete, and tumor subclones that escape the requirement for the initiating oncogene can recur (Figure 9-5; 52). Interestingly, in a model of MYC-induced hepatocellular carcinoma, MYC reactivation after tumor regression results in the development of tumors that are clonally related to the initial primary tumor, indicating that dormancy can also be a result of oncogene inactivation (23). These dramatic results validate the future use of these models to predict the outcome of altering specific pathways predicted to influence tumor survival or progression. Clinically, pharmacologic oncogene inactivation can successfully reduce tumor growth supporting the concept of oncogene addiction. In particular, a subset of non-small cell lung cancer with mutant EGFR expression (56,57), gastrointestinal stromal tumors with active/mutant c-Kit (58), and chronic myeloid leukemia with the BCR-ABL translocation (59) have been successfully treated with targeted small molecules.

Oncogene Cooperation and Codependence

The hypothesis of a multistep model of tumorigenesis mediated by multiple genetic alterations and the discoveries that validated this model raised the interesting question of how these genes cooperate to promote tumor development. In vitro studies in immortalized cell lines and primary fibroblasts were initially used to show the cooperativity of different oncogenes (60). The tumor suppressor networks, the relationship between oncogenes and their target genes, and the cooperation of different genetic changes in promoting tumor initiation and progression have also been studied in vivo using genetic methods (61–63). Genetic epistasis experiments in mice have identified several critical targets of specific oncogenes that mediate different aspects of tumorigenesis (31,61,64). Moreover, genes that enhance or reduce the effect of tumor suppressor gene mutation and oncogene expression have also been identified (62,63,65). Overall, these studies have allowed our understanding of tumor signaling to move from a reductionist to a systems biology level.

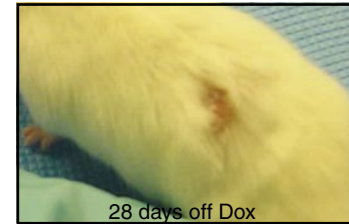
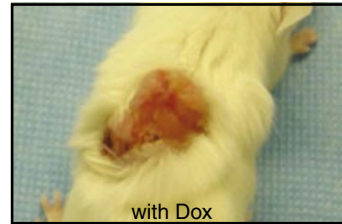
Future Directions of Cancer Models

The most advanced genetically engineered mouse models of human cancer reflect their human counterparts at the genetic and histologic levels. These mouse models are now poised to lead the way to the discovery of new genes and pathways dysregulated in cancer and aid in the development and screening of potential therapeutics.

In Vivo Screens

Transposon and retroviral insertional mutagenesis, short-interfering RNA (siRNA) libraries, and advances in the analysis of gene expression and genomic alteration will allow mouse models to move to the forefront as tools in the discovery of new cancer genes and pathways.

Tyr-rtTA;tetO-Ras^{G12V};Ink4a/Arf^{-/-}



CCSP-rtTA;tetO-EGFR^{L858R}

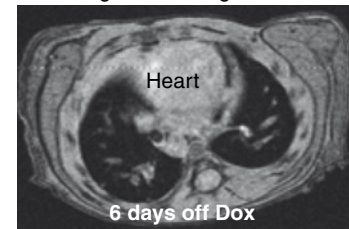
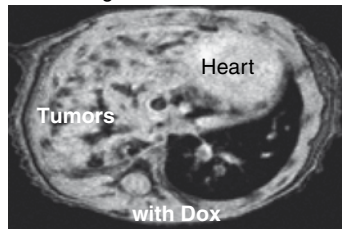


FIGURE 9-5 Oncogene addition of mouse tumors predicts the outcome of therapeutic blockade of oncogene expression or activity. Melanocyte-specific oncogenic Ras expression leads to the formation of melanoma, which regresses when Ras is no longer expressed. Lung epithelial expression of an active point mutant of EGFR produces lung adenocarcinoma development. The maintenance of these lung tumors relies on the continued expression of the oncogene. (Melanoma images are courtesy of Joseph Hyeon Nam Jeong and Lynda Chin, Dana-Farber Cancer Institute, Harvard University. Lung adenocarcinoma images are courtesy of Katerina Politi and Harold Varmus, Memorial Sloan-Kettering Cancer Center.)

Each of these approaches has been used to identify genes that promote tumorigenesis (66–75). Unlike chemical or physical mutagens, insertional mutagens allow the identification of mutated genes. By using these mutagens in sensitized backgrounds (for example loss of a tumor suppressor or expression of an oncogene), the genes that regulate tumor initiation, invasion, or metastasis can be identified. siRNA library screens for genes that influence transformation have been conducted in vitro (70–72) and the prospect of focused or genome-wide siRNA screens in vivo is alluring. Genes discovered by these methods can be confirmed in the same tumor model in which they are found and these unbiased approaches may identify genes and pathways that are potential therapeutic targets.

Validation of Pharmaceutical Targets and Preclinical Trials

The development of new therapeutics requires carefully designed preclinical studies in models that most closely approximate human

disease. Xenograft tumor models are the mainstay of current pre-clinical testing. While several obstacles must be overcome before genetic mouse models can fully reach their potential in pharmacologic and biotechnological settings, these models may more accurately reflect the therapeutic response of patients (7,8). The use of genetically defined mouse models may prioritize potential therapeutic compounds and accelerate their translation into the clinic.

Biomarkers for Early Tumor Detection

The detection of cancer at an early stage is of paramount importance, as patients diagnosed with early-stage disease invariably have a better prognosis. Unfortunately, there is a dearth of sensitive and reliable screening tests for most solid tumors. Here again, mouse models on inbred backgrounds with controllable and reproducible disease, coupled with advances in proteomic and molecular imaging technologies, may allow new diagnostic markers to be identified.

Identification of the Cell of Origin

Spatial and temporal restriction of genetic alterations in mice also allow the initial events that are triggered by oncogene expression to be investigated and the cells that respond to these initial genetic lesions to be identified. Specific genetic manipulation in defined cell types can identify the cell type in a given tissue that is susceptible to oncogenic transformation (76). Alternatively, analyzing the cells that respond after in vivo oncogene activation may identify the cells of origin. This technique putatively identified bronchioalveolar stem cells as the cell of origin in a mouse model of non-small cell lung cancer (77). The appeal of these approaches is not solely to identify tumor initiating cells but also to allow their subsequent manipulation and the identification of critical pathways dysregulated in these cells.

Recruitment and Function of Immune, Vascular, and Stromal Cells in the Tumor Environment

It has become increasingly clear that tumor growth and progression is greatly influenced by surrounding nontumor cells includ-

ing various immune cell types, vascular cells, stromal fibroblasts and myofibroblasts (78,79). Mouse models in which each of these tumor cell populations can be manipulated independently will allow the function of each cell type to be identified. Moreover, molecules that regulate the recruitment, survival, and function of these cells within the tumor can be characterized in mouse models in vivo. The secreted and cell surface molecules used by these cells to communicate with each other and with the tumor cells will lead to the identification of important regulators of tumor growth, angiogenesis, invasion, and metastasis.

Conclusions

Genetically engineered mouse models of human cancers are an important component of the arsenal of experimental systems that will allow the in vivo dissection of tumor biology over the next several decades. The versatility of mouse models that recapitulate human cancer will lead to timely identification and validation of therapeutic targets that will ultimately influence human health.

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Cancer Stem Cells

Not All Cancer Cells are Created Equal

Much of clinical oncology is founded on the assumption that most cancer cells are capable of proliferating indefinitely and of disseminating potentially fatal malignancies. As a result, therapies for most types of cancer are designed to eliminate all cancer cells. However, it has long been recognized that cancer cells within individual tumors are phenotypically heterogeneous (1–3) despite being clonally related (4–6). This raises the question of whether the phenotypic differences among cancer cells within a single tumor are associated with functional differences. Are some cancer cells more malignant than others?

Evidence demonstrates that this is true in certain cancers including testicular cancer (7), acute myeloid leukemia (8,9), breast cancer (10), certain brain cancers (11,12), and colon cancer (99,100). In each of these cancers, most of the cancer cells appear to lack the ability to proliferate extensively or to transfer disease on transplantation. In contrast, a phenotypically distinct minority of cancer cells is highly enriched for cells that can proliferate extensively and transfer disease. These cells have been termed “cancer stem cells” because, like normal stem cells, they appear to self-renew (forming more cancer stem cells than can be serially transplanted among immunocompromised mice) and give rise to phenotypically diverse nontumorigenic cancer cells that compose the bulk of the tumors they form (Figure 10-1; 13,14). If these cancer stem cells are uniquely capable of forming new tumors, as suggested by the data, then to cure these cancers it will be necessary and sufficient to kill the cancer stem cells.

While cancer stem cells are often phenotypically and functionally similar to normal stem cells from the same tissue (Table 10-1), the cancer stem cell model does not imply that cancer stem cells must arise from normal stem cells (15–17). The cancer stem cell model describes the observation that cancer cells are heterogeneous and exist within a hierarchy of proliferative potentials, regardless of whether the cancer stem cells arise from the transformation of normal stem cells, downstream-restricted progenitors, or differentiated cells (Figure 10-2; 13,14). In reality, many cancer stem cells are likely to arise from the transformation of normal stem cells as normal stem cells are the only mitotic cells that persist long enough in many tissues to accumulate the mutations necessary for transformation (13). However, mutations in restricted progenitors can confer upon those progenitors self-renewal potential and cancer stem cell properties (18,19). As a result, the mere existence of cancer stem cells does not address the origin of these cells: Some cancer stem cells likely arise from

the transformation of normal stem cells, whereas other cancer stem cells arise from restricted progenitors or differentiated cells that have acquired stem cell properties via mutagenesis.

Not a New Idea

The idea that cancer growth and progression may be driven by a minority population of cancer stem cells is an old one (20). This idea arose from the observation that in a wide variety of malignancies only a small proportion of cancer cells were able to proliferate extensively, regardless of how proliferative potential was assayed. For example, when mouse myeloma cells were separated from normal hematopoietic cells and put in clonal, in vitro colony-forming assays only 1 in 10,000 to 1 in 100 cancer cells were able to form colonies (21). Even when leukemic cells were transplanted in vivo, only 1% to 4% of cells were able to form spleen colonies (22–24). The clonogenic leukemia cells were described as leukemic stem cells (21); however, the observation that only a minority of leukemic cells proliferated in these assays left two possible explanations. One possibility was that all leukemia cells had the same low probability of proliferating extensively in these assays. The second possibility was that most leukemia cells were unable to proliferate extensively and that only a small, definable subset of cells was consistently clonogenic (Figure 10-1).

To distinguish between these possibilities, it was necessary to separate different classes of leukemia cells and test whether some cells were more clonogenic than others. In the 1960s and 1970s, technology for cell separation was not up to this task and, as a result, the cancer stem cell model was never formally proven for most cancers and fell out of favor. However, with the advent of flow cytometry, it became possible to precisely separate phenotypically distinct populations of cells on the basis of differences in cell surface marker expression, cell size, DNA content, and other characteristics (Figure 10-3). Using flow cytometry to separate phenotypically distinct subsets of acute myeloid leukemia cells, John Dick and colleagues showed that most human acute myeloid leukemia cells were unable to transfer disease upon transplantation into immunocompromised mice, whereas a small subpopulation of CD34⁺CD38[−] leukemia cells was highly enriched for the ability to transfer disease (8). This formally proved that leukemias were organized in

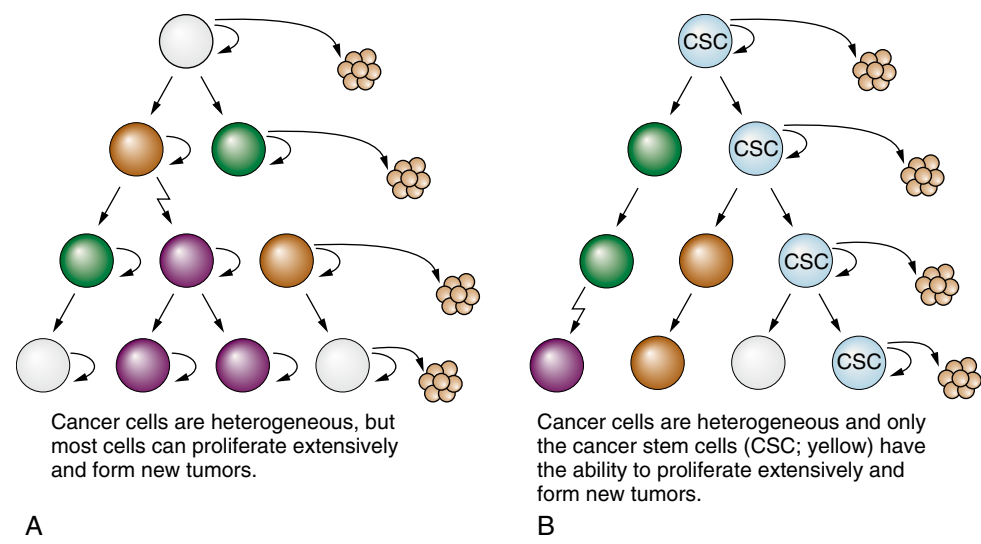


FIGURE 10-1 THE CANCER STEM CELL MODEL. Two general models of solid cancer cell heterogeneity can be summarized as the stochastic model (A) and the cancer stem cell model (B). In the stochastic model (A), cancer cells of many different phenotypes have a similar potential to proliferate extensively but each single cell has a low probability of actually exhibiting this potential. In the cancer stem cell model (B), most cancer cells have only limited proliferative potential, but a subset of cancer cells consistently proliferates extensively in clonogenic assays and can form new tumors upon transplantation. These cells have been termed “cancer stem cells” (CSCs; yellow) based on their ability to self-renew (forming additional cancer stem cells that can be serially transplanted) and form phenotypically diverse progeny (nontumorigenic cancer cells) with limited proliferative potential. Strong evidence supports the cancer stem cell model in teratocarcinoma, acute myeloid leukemia, breast cancer, colon cancer, and certain brain cancers. However, it remains uncertain whether most cancers follow a cancer stem cell model or a stochastic model. Although existing anticancer therapies have largely been predicated on the idea that it is necessary to eliminate every cancer cell (A), the cancer stem cell model predicts that it is necessary and sufficient to eliminate the cancer stem cells (B). (From *Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105, with permission.*)

Table 10-1 Normal Stem Cell Properties Versus Cancer Stem Cell Properties

Normal Somatic Stem Cells	Cancer Stem Cells
Extensive but limited self-renewal capacity	Extensive and indefinite self-renewal capacity
Organogenic capacity	Tumorigenic capacity
Capacity to generate differentiated progeny with limited proliferative potential, often phenotypically diverse	Capacity to generate abnormal progeny with limited proliferative potential, often phenotypically diverse
Highly regulated self-renewal and differentiation	Highly dysregulated self-renewal and differentiation
Rare in normal adult tissues	Infrequent or rare within tumors
Identifiable based on surface markers	Often express similar surface markers as normal stem cells in the same tissue
Normal karyotype	Abnormal karyotype
Quiescent most of the time	Less mitotically active than other cancer cells

a hierarchical manner, just like normal hematopoiesis, with a small population of stem cells that gave rise to a larger population of phenotypically diverse cells with limited proliferative potential (see following sections for more details). The ability to consistently identify the leukemic stem cell population by selecting CD34⁺CD38[−] cells, even in patients with different subtypes of acute myeloid leukemia, demonstrated that phenotypically similar leukemic stem cells arose in cancers with different underlying mutations.

Like leukemia cells, solid cancer cells are also phenotypically heterogeneous and include only a small proportion of cells that are clonogenic in culture and in vivo (1–3,25,26). For example, it has long been recognized that less than 1% of lung cancer, ovarian cancer, or neuro-

blastoma cells form colonies in soft agar (20). Like John Dick’s work in acute myeloid leukemia, research has demonstrated that not all human breast cancer cells (10) or brain cancer cells (11,12) are equal in their ability to proliferate. In both cases, most cancer cells had a limited ability to proliferate in vitro or in vivo, whereas a phenotypically distinct subpopulation of cells from multiple patients was highly enriched for the ability to form tumors on transplantation into immunocompromised mice (see following sections for more details). These observations ruled out the possibility that all breast cancer or brain cancer cells had a similar clonogenic capacity and demonstrated that a small, phenotypically distinct subset was consistently highly enriched for the ability to form new tumors relative to the bulk population of cancer cells.

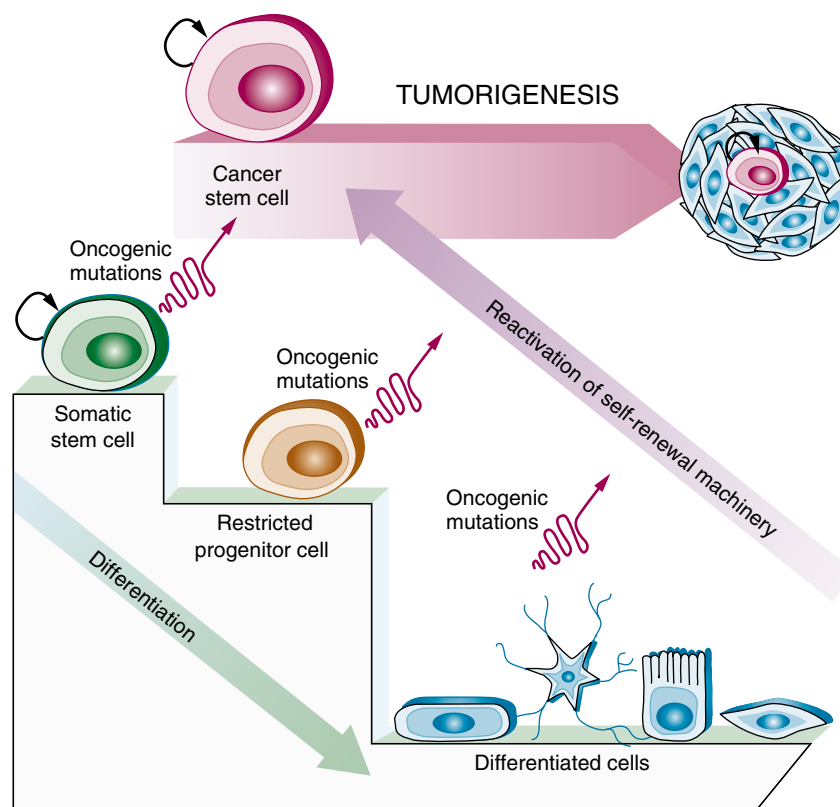


FIGURE 10-2 THE CELLULAR ORIGIN OF CANCER STEM CELLS. In principle, cancer stem cells could arise from mutations that transform either normal somatic stem cells, or downstream restricted progenitors, or even differentiated cells. In each case, the mutations would have to confer upon these cells a dysregulated ability to self-renew as well as tumorigenic potential. In practice, some cancers likely arise from each of these routes. However, stem cells may be more likely than other somatic cells to accumulate the mutations necessary for transformation because these cells are longer lived than other mitotic cells in most tissues, and may require fewer mutations for transformation since self-renewal pathways are already active in these cells. Even in cancers in which cancer stem cells have been identified, it remains uncertain whether they arose from normal stem cells, restricted progenitors, or differentiated cells.

Testicular Cancer Follows a Cancer Stem Cell Model

Although the cancer stem cell model has been regarded as a new way of understanding the growth and progression of cancer, testicular cancer has been understood to follow a cancer stem cell model since the 1960s (7). Germ cell tumors in the testis are malignant and contain highly proliferative, undifferentiated teratocarcinoma cells that give rise to postmitotic differentiated cells of all three germ layers, such as neurons, hair, bone and muscle. Upon serial transplantation, single undifferentiated teratocarcinoma cells can give rise to new tumors that again contain proliferative undifferentiated cells along with diverse types of differentiated cells (7). Moreover, teratocarcinoma cells are pluripotent: When single cells derived from mouse testicular teratocarcinomas are injected into embryos, they can undergo pluripotent differentiation, contributing to all tissues including the germ line (27–30). These chimeric animals can develop into normal healthy adults with few or no tumors, despite the widespread contribution of teratocarcinoma cells to their tissues. This demonstrates that the differentiated cells that arise from malignant teratocarcinoma stem cells are benign despite carrying the same mutations that cause neoplastic proliferation by the undifferentiated cells (31). Indeed, unresectable differentiated

cells can persist in testicular cancer patients after chemotherapy for years without resuming division (32). These characteristics of teratocarcinoma have long been regarded as strong evidence in support of the cancer stem cell model and proof that at least some types of malignant cells can differentiate into phenotypically diverse benign cells (7).

Teratocarcinomas are not unique among solid cancers in manifesting evidence of differentiation. Differentiation of cancer cells into progeny with characteristics of mature cells is also evident to various degrees in a number of other cancers (33,34). However, in most of these cases, it has not been clear whether the cells that express differentiation markers become postmitotic or whether they are truly benign. As a result, teratocarcinoma has been regarded as unusual in its adherence to a cancer stem cell model in which tumor growth and progression is clearly driven by a minority population of undifferentiated cells. Nonetheless, more recent advances in the context of other cancers have demonstrated that many cancers may follow a cancer stem cell model, even in cases where differentiation is not necessarily evident within tumors. This implies that cancer cells, like normal progenitors, may frequently be capable of undergoing spontaneous epigenetic changes that limit their proliferative potential regardless of whether they manifest overt markers of differentiation.

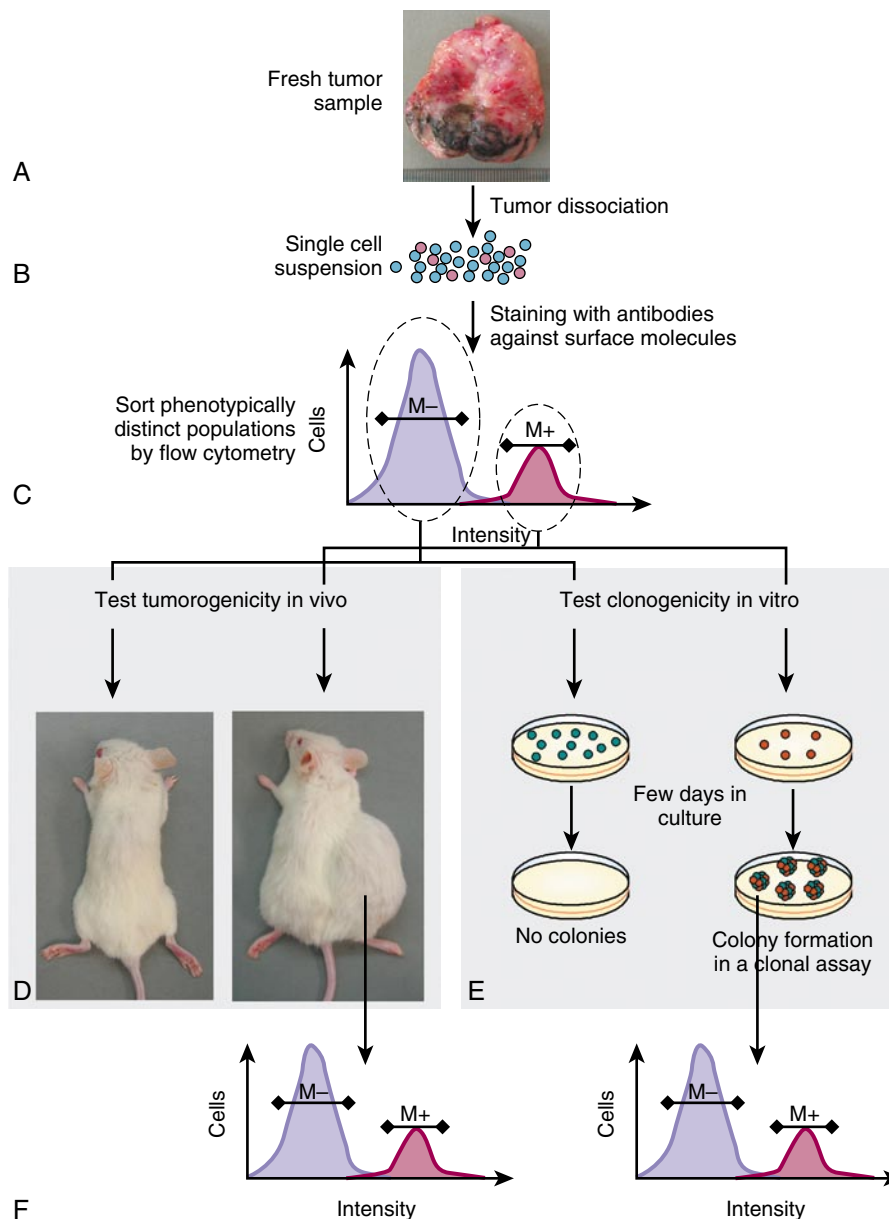


FIGURE 10-3 PROSPECTIVE IDENTIFICATION OF CANCER STEM CELLS. To demonstrate that some cancer cells are enriched for tumorigenic potential, whereas other cancer cells are depleted for tumorigenic potential, it is necessary to separate phenotypically distinct subsets of cancer cells and then assay their tumorigenic and clonogenic potentials separately. This can be done using freshly obtained cells from hematopoietic malignancies or enzymatically dissociated solid tumors (A). A single-cell suspension of cancer cells (B) is stained with antibodies to identify surface markers that allow the separation of cancer cells into phenotypically distinct fractions. These fractions are then separated by flow cytometry (C) to test whether they differ in functional assays. Common assays involve comparing the ability of various doses of cancer cells to form tumors in immunocompromised mice (D) or to proliferate in culture (E). If a phenotypically distinct fraction of cancer cells (M+; red) is consistently enriched for tumorigenic potential in vivo and clonogenic potential in culture compared with the bulk cancer population, whereas other cancer cells from the same tumor (M-; blue) exhibit little or no ability to form tumors in vivo or colonies in culture, then the M+ cells may include cancer stem cells. Cancer stem cells also regenerate the phenotypic diversity present in the original tumor, including additional M+ cancer stem cells as well as M- nontumorigenic cancer cells (F).

Acute Myeloid Leukemia

Dick and colleagues were the first to prove that acute myeloid leukemia followed a cancer stem cell model. They found that $CD34^+CD38^-$ cells that represent only 0.2% to 1% of all leukemia cells were the only cells that were capable of transferring disease on transplantation into immunocompromised NOD/SCID mice (8,9). Most $CD34^-$ and/or $CD38^+$ leukemia cells

from the same patients were unable to transfer disease. Moreover, the $CD34^+CD38^-$ leukemic stem cells were consistently highly enriched for leukemogenic activity in multiple patients with multiple different subtypes of acute myeloid leukemia. This demonstrates that acute myeloid leukemia is hierarchically organized with small numbers of leukemic stem cells that give rise to more phenotypically diverse leukemia cells with limited proliferative potential. Leukemic stem cells probably form non-leukemogenic progeny by undergoing epigenetic changes akin

to the differentiation of normal stem cells. Thus, vestiges of the differentiation programs that are operative in normal stem cells may persist in cancer cells despite the presence of transforming genetic mutations.

Leukemic stem cells exhibit phenotypic similarities to normal hematopoietic stem cells. Normal hematopoietic stem cells are also $CD34^+CD38^-$ (35–38). Moreover, both normal hematopoietic stem cells (39,40) and leukemic stem cells (41,42) are relatively quiescent. This suggests that leukemic stem cells divide infrequently but constantly to form more proliferative leukemia cells that divide for a limited period of time to increase leukemic burden before becoming exhausted. There are also phenotypic differences between leukemic stem cells and normal hematopoietic stem cells, including differences in the expression of Thy-1 (on normal but not leukemic stem cells; 43), c-kit (on normal but not leukemic stem cells; 44), and interleukin-3 (IL-3) receptor (on leukemic but not normal stem cells; 45).

The overall phenotypic similarity between leukemic stem cells and normal hematopoietic stem cells has caused some to hypothesize that leukemic stem cells arise from normal hematopoietic stem cells. This is a plausible hypothesis as in contrast to hematopoietic stem cells, myeloid restricted progenitors, and differentiated myeloid cells have very short half-lives and little opportunity to accumulate the mutations required for transformation (13). Nonetheless, oncogenic mutations can confer cancer stem cell properties on restricted myeloid progenitors (18,19), raising the possibility that initial mutations may accumulate in hematopoietic stem cells but the final transforming mutation might sometimes occur in downstream-restricted progenitors (15,16).

Leukemic stem cells are also functionally similar to normal hematopoietic stem cells. In general, tumor suppressors that inhibit cancer cell proliferation frequently inhibit stem cell self-renewal in the same tissues, while proto-oncogenes that promote cancer cell proliferation also promote stem cell self-renewal (13,46,47). A particularly striking example of this mechanistic parallel comes from Bmi-1, a polycomb family transcriptional repressor (48). Bmi-1 is required for the self-renewal of every type of adult stem cell examined (49–53) without being generically required for the proliferation of all cells (50). Bmi-1 appears to play a very similar role in leukemic stem cells and in hematopoietic stem cells in that it is not required for the formation or differentiation of either type of cell, but is required for the maintenance of both cell types upon serial transplantation (51). The similarities in mechanisms that regulate the self-renewal of leukemic stem cells and normal hematopoietic stem cells imply that oncogenic transformation hijacks the normal self-renewal machinery to confer neoplastic potential on normal cells.

Evidence supports the possibility that other hematopoietic malignancies also follow a cancer stem cell model. For example, most acute B-lymphoblastic leukemia (B-ALL) cells express the B-cell markers CD10 and CD19. However, in one study the cells that could proliferate long term in culture and transfer disease to immunocompromised NOD/SCID mice were $CD34^+CD10^-$ or $CD34^+CD19^-$, populations that account for only a few percent of acute lymphoblastic leukemia cells in patients with at least certain forms of the disease (54). Engraftment of the $CD34^+CD10^-$ cells in mice was 30- to 100-fold more efficient than engraftment

of unfractionated cells. Moreover, the engrafted cells displayed the same phenotype as the bulk acute lymphoblastic leukemia population and could be retransplanted into secondary recipients (54). Another study that also concluded that acute lymphoblastic leukemia follows a cancer stem cell model found that the leukemia-initiating cells were $CD34^+CD38^-CD19^+$, therefore expressing at least one marker of differentiated B cells (CD19) (101). The leukemia-initiating cells may acquire somewhat different phenotypes in response to different underlying mutations. These observations suggest that in acute lymphoblastic leukemia, like in acute myeloid leukemia, infrequent immature cells sustain the leukemia by giving rise to differentiated lymphoblastic cells that represent most leukemia cells but which themselves have limited replicative potential. Overall, much additional work will be required to rigorously test the extent to which different types of hematopoietic malignancies follow a cancer stem cell model.

Breast Cancer

Many had regarded teratocarcinoma and hematopoietic malignancies as being unusual in their adherence to a cancer stem cell model and believed that other solid cancers that showed less obvious differentiation would be composed of cells with more uniform tumorigenic potential. However, research findings indicate that human breast cancer also follows a cancer stem cell model, with a small fraction of tumorigenic breast cancer stem cells and a larger, phenotypically diverse population of breast cancer cells that lacks tumorigenic potential (10). In this study, uncultured specimens of breast cancer cells from nine patients were fractionated by flow-cytometry based on the differential expression of adhesion molecules and injected into the mammary fat pads of immunodeficient NOD/SCID mice (10). A small subpopulation of the tumor cells (generally composing fewer than 10% of tumor cells) that expressed CD44 (the hyaluronate receptor) but failed to express high levels of CD24 (a ligand for P-selectin) was highly enriched for tumorigenic cells. As few as 100 of these $CD44^+CD24^{-/low}$ cells were able to form a tumor. In contrast, tens of thousands of tumor cells that were $CD44^-$ and/or $CD24^{high}$ failed to form tumors. Importantly, these nontumorigenic cells did include cancer cells. The $CD44^+CD24^{-/low}$ population was enriched for tumorigenic potential relative to unfractionated tumor cells in eight of nine patients, including a primary tumor and several metastatic tumors. This suggests that like in leukemia, tumorigenic breast cancer cells are highly enriched within a distinct population of breast cancer cells that expresses markers that are widely conserved among patients.

Historically, heterogeneity within tumors has been thought to arise largely from ongoing genetic changes that cause cancer cells to become phenotypically and functionally different as they accumulate additional mutations. Although ongoing genetic change certainly contributes to cancer progression (55), it has never been clear that genetic change occurs rapidly or pervasively enough to account for the widespread heterogeneity within individual tumors. In the case of breast cancer, if genetic differences accounted for the phenotypic and functional differences between tumorigenic $CD44^+CD24^{-/low}$ cells and nontumorigenic cells,

secondary tumors that arise from the $CD44^+CD24^{-/low}$ population would be expected to be composed of expanded numbers of tumorigenic $CD44^+CD24^{-/low}$ cells, rather than the range of phenotypes represented in primary tumors. However, tumors that arose from the transplantation of $CD44^+CD24^{-/low}$ cells contained both tumorigenic $CD44^+CD24^{-/low}$ cells as well as nontumorigenic $CD44^-$ and/or $CD24^{high}$ cells (10). Thus the $CD44^+CD24^{-/low}$ population regenerated tumors that mimicked the phenotypic diversity present in the original tumor. These results strongly suggest that this phenotypic diversity present within breast tumors arises via the differentiation of breast cancer stem cells into nontumorigenic breast cancer cells. Heterogeneity within tumors thus arises from both epigenetic and genetic changes within the cancer cells, although the nature of these epigenetic changes is just beginning to be studied, in contrast to genetic changes that have already been extensively characterized in many cancers.

Brain Cancers

The cancer stem cell model also applies to some cancers of the nervous system, such as medulloblastoma, gliomas, and ependymomas (11,12,56). In each of these cases, a minority subpopulation of cancer cells with phenotypic and functional features of neural stem cells appears uniquely capable of proliferating in culture and forming tumors in vivo. The evidence in support of this conclusion is based partly on the ability of these brain cancer stem cells to form neurospheres in culture, in contrast to the majority population of brain cancer cells that failed to proliferate in culture (Figure 10-4). Neurosphere formation is a commonly used assay for neural stem cells in which neural cells are added at low cell density to nonadherent cultures in the presence of mitogens such as epidermal growth factor and fibroblast growth factor (57). Under these conditions,

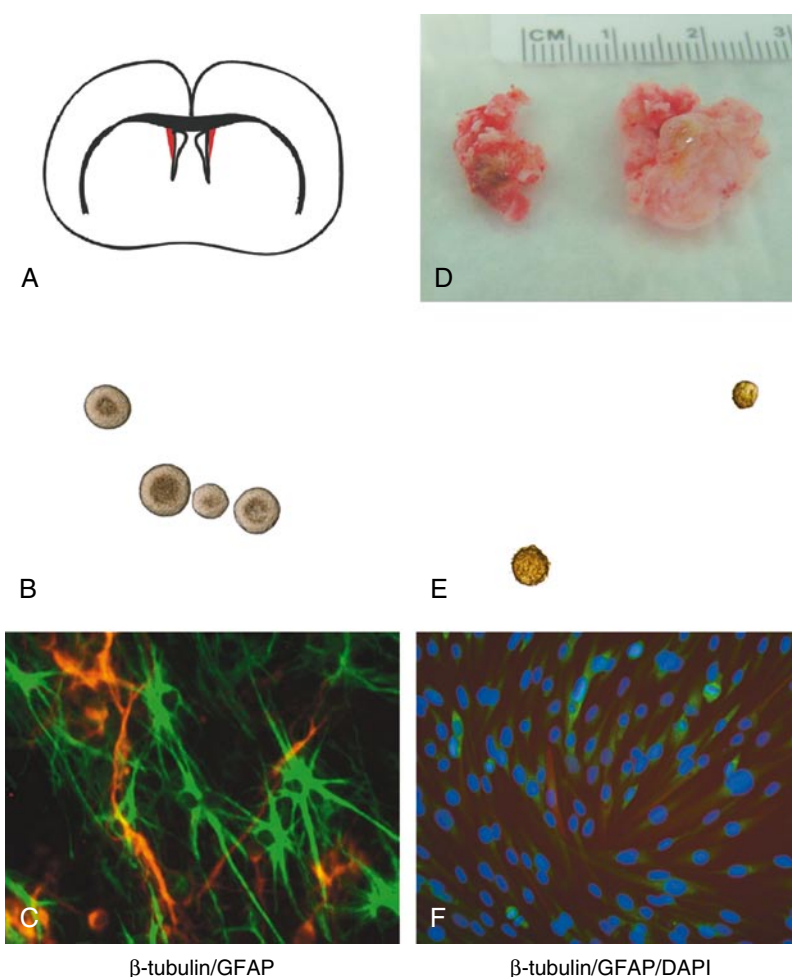


FIGURE 10-4 CANCER CELLS FROM HUMAN GLIOMAS CAN FORM NEUROSHERES IN CULTURE, SUCH AS NORMAL NEURAL STEM CELLS. A few percentage of cells from the subventricular zone in the lateral wall of the lateral ventricle of the mouse and human brains (A, red) can form colonies called neurospheres (B) in nonadherent cultures. When cells are plated at very low cell densities, these neurospheres arise from single cells but grow to contain thousands of cells, including neural stem cells and restricted neuronal and glial progenitors. If the neurospheres are replated to adherent cultures and mitogen concentrations are reduced, neurosphere cells undergo multilineage differentiations (C) to form neurons (red), astrocytes (green), and oligodendrocytes (not shown). Human gliomas (D) and other brain tumors contain a minority population of brain cancer stem cells that can also form neurospheres in culture (E). On transfer to adherent cultures, these cancer neurospheres can also give rise to progeny that express differentiation markers (F). Sometimes these cancer neurospheres undergo multilineage differentiation, but more often they undergo aberrant differentiation that reflects the markers expressed in the tumor of origin. In this case, the cancer neurosphere gave rise mainly to cancer cells that expressed a glial marker (green), but only a few cells that weakly expressed a neuronal marker (red).

individual neural stem cells proliferate to form free-floating spheres of neural progenitors termed “neurospheres,” which are capable of undergoing multilineage differentiation with transfer to adherent cultures and mitogen withdrawal. These primary spheres can then be dissociated mechanically or enzymatically and reseeded into secondary cultures where individual stem cells will again form “secondary” spheres (self-renewal). This assay can thus be used to measure the self-renewal and differentiation of individual neural stem cells in culture.

Based on this assay, a variety of brain tumors was shown to contain a minority population of CD133⁺ (a glycosylated form of a cholesterol-binding, cell-surface molecule called prominin) cancer cells that could form neurospheres in culture, in contrast to most CD133[−] cancer cells that failed to proliferate in culture (11,12,56). Some normal human neural stem cells also express CD133 (58). The CD133⁺ cells also express nestin, another marker of neural stem cells, whereas the CD133[−] cells often express markers of differentiated neurons or glia. Beyond CD133 and nestin expression, brain cancer stem cells are also remarkably similar to normal neural stem cells from the brain in that they can be serially passaged in culture and undergo multilineage differentiation in some cases. Nonetheless, neurospheres derived from brain cancer cells can be passaged for much longer than normal human neural stem cells and often differentiate to neurons and glia in proportions that reflect what is observed in the original tumors. This suggests that the phenotypic heterogeneity observed within brain tumors is largely caused by epigenetic changes that occur as brain cancer stem cells differentiate to form cancer cells with properties similar to neurons and glia.

Consistent with the difference in proliferative potential in culture, CD133⁺ cells were also enriched for the ability to form brain tumors upon transplantation into immunodeficient mice as compared with unfractionated brain tumor cells or CD133[−] brain tumor cells (56). Injection of as few as 100 CD133⁺ cells into the brains of immunodeficient mice led to the formation of a new tumor that recapitulated the histologic features of the primary tumor from which they derived. These secondary tumors contained additional CD133⁺ cells as well as a majority population of CD133[−] cancer cells, both displaying an abnormal karyotype. The CD133[−] brain tumor cells were greatly depleted for the ability to form brain tumors (tumors failed to form even when mice were injected with much larger numbers of cells), despite the fact that these cells carried the same mutations as observed in the CD133⁺ cells from the same tumors (56). These observations indicate that brain cancer cells within a single tumor are frequently heterogeneous in proliferative potential, with the clonogenic cells often exhibiting features that are similar to normal neural stem cells.

Although brain cancer stem cells exhibit properties that are similar to normal neural stem cells, it is not clear whether the cancer stem cells arise from normal stem cells or other cells. Neural stem cells appear to persist throughout adult life in certain regions of the human brain (59,60). The accumulation of mutations in these cells could transform them into cancer stem cells. Alternatively, cancer stem cells might arise from the transformation of other cells, like parenchymal glia, that could dedifferentiate to acquire properties similar to neural stem cells after acquiring mutations (61). Cancer stem cell properties may differ depending on cell of origin and the combination of transforming mutations that they carry.

It is not known whether the differentiation of tumorigenic, CD133⁺ brain cancer stem cells into CD133[−] nontumorigenic brain cancer cells is irreversible. The observation that the CD133⁺ cells are orders of magnitude more tumorigenic than the CD133[−] cells demonstrates that in the cancers studied so far the CD133[−] cells do not efficiently revert to a tumorigenic CD133⁺ phenotype. Nonetheless, it remains possible that the epigenetic changes that distinguish CD133⁺ cells from CD133[−] cells are reversible in other cases, at least in certain types of brain cancer or in cases that are caused by certain mutations. Thus it remains possible that certain types of brain cancer may not follow a cancer stem cell model, particularly if there is efficient interconversion between the undifferentiated and differentiated subsets of cells.

Even if differentiated brain cancer cells can only revert to tumorigenic cells with very low efficiency, this might still have clinical implications for therapies that target brain cancer stem cells. Any therapy for brain cancer that specifically targets the dividing, undifferentiated brain cancer stem cells would leave behind differentiated brain cancer cells. If these differentiated cells can revert to a tumorigenic phenotype with even a low efficiency, these tumors would recur after therapy. In testicular cancer, the differentiated cancer cells that are left behind after chemotherapy usually do not resume division or conceal the presence of proliferating cancer cells. Nonetheless, they do so frequently enough that resection of even differentiated cancer cells is recommended (62). In the brain, the complete resection of tumors can be impossible due to their diffuse, invasive nature or due to their proximity to critical brain regions. For these reasons, even the ability to efficiently target brain cancer stem cells might not lead to cures. This is a potential issue for all therapies that target cancer stem cells and will have to be studied on a case-by-case basis as the efficiency with which differentiated cells revert to a tumorigenic phenotype may vary according to the type of cancer, the cell of origin, and the mutations involved.

Could Cancer Stem Cells be an Artifact of the Assays that have been Used to Identify them?

The strongest evidence in support of the cancer stem cell model comes from experiments in which human cancer cells have been tested for tumorigenicity after transplantation into immunocompromised mice. This raises the formal possibility that some cancer cells that are tumorigenic in humans may not be tumorigenic in mice because of physiologic differences between the species or the xenogeneic immune response that occurs against human cells (even in immunocompromised mice). Consistent with this possibility, mouse leukemia-initiating cells that were detected based on histocompatible transplants into syngeneic recipient mice were much more frequent and phenotypically diverse than human leukemia-initiating cells detected upon transplantation into immunocompromised mice (102, 103). This raises the possibility that we may be significantly underestimating the frequency of human cancer stem cells in immunocompromised mice, or that we may be detecting only the most tumorigenic subset of these cells. Nonetheless,

the evidence that supports the cancer stem cell model in the cases described previously is unlikely to be an artifact of this xenogeneic assay for several reasons. First, it is well documented that differentiated cancer cells that arise from teratocarcinoma in patients usually do not resume division after the elimination of undifferentiated cells by chemotherapy (see testicular cancer in preceding sections). This proves that at least some cancer cells can become postmitotic in the patients in which the cancers arise. Second, even when other solid cancer cells, like ovarian cancer, have been transplanted from their site of origin to a second subcutaneous site in the same patient, large numbers of cells have been required to form a new tumor, consistent with the idea that only a fraction of cancer cells is tumorigenic (25). Thus, few cancer cells appear tumorigenic even when autotransplanted in patients. Third, even when the clonogenicity of hematopoietic or solid cancer cells has been tested in culture, the evidence has consistently indicated that only a minority of cancer cells is clonogenic. Brain cancer provides the most compelling example, where the CD133⁻ cancer cells are consistently less clonogenic in culture than CD133⁺ cancer cells (11,12,56). These observations make it unlikely that the evidence supporting the cancer stem cell model is entirely an artifact of xenogeneic transplantation.

Although it is clear that some cancer cells are more clonogenic than others, some of the finer points of the cancer stem cell model may still be influenced by the experimental systems in which it is tested. For example, when human cancer stem cells have been identified by transplantation into immunocompromised mice, it has generally been concluded that the cancers were sustained by a single, phenotypically distinct cancer stem cell population that was uniquely capable of proliferating extensively. However, when the cancer stem cell model was tested in mice engineered to develop acute myeloid leukemia after *Pten* deletion, individual mice showed evidence of multiple, phenotypically distinct cancer stem cell populations (63). In these mice, the leukemic stem cell model was tested by transplanting various fractions of hematopoietic cells into fully histocompatible mice, which were then monitored for the development of leukemia. Just as observed in human acute myeloid leukemia (8,9), mouse leukemia cells that expressed hematopoietic stem cell markers were orders of magnitude more enriched for leukemia-initiating activity than unfractionated bone marrow or leukemic blast cells (63). Nonetheless, cells that expressed mature myeloid markers were still able to transfer disease, albeit much less efficiently. This raises the possibility that cancers contain multiple, phenotypically distinct clonogenic populations at different stages of a hierarchy.

There is some experimental support for the idea that human leukemic stem cells are also heterogeneous in their potential to transfer disease as serial transplantation has revealed differences among leukemic stem cells in their ability to transfer disease to secondary and tertiary mouse recipients (64). This supports the idea that some leukemic stem cells are more potent than others, although it does not provide evidence that leukemia cells expressing mature myeloid markers can transfer disease as observed in the experiments on *Pten*-deficient mice (63). The failure to detect multiple phenotypically distinct cancer stem cells within individual human tumors could be caused by the xenogeneic immune response in immunocompromised mice that rejects all but the most potent tumorigenic cells. Perhaps rather than having no tumorigenic activity, CD133⁻

human brain cancer cells that express neuronal or glial markers have low levels of tumorigenic activity that cannot readily be detected in mice. Such a possibility would not challenge the central inference in the cancer stem cell model—that some cancer cells are much more tumorigenic than others. However, it would raise the possibility that it will not be sufficient to target only cancer stem cells. Additional research will be required to investigate these issues because there are other potential explanations for this inconsistency between mouse and human results, including that cancer stem cells are phenotypically unstable and that *Pten*-deficient mice develop polyclonal leukemias, each having a distinct cancer stem cell population.

Other technical issues must also be considered carefully in evaluating the cancer stem cell model. Particularly if cells are enzymatically dissociated before flow cytometry, does the dissociation remove surface markers and damage a subset of cells in a way that renders them unable to survive or unable to form tumors after transplantation? Is the nontumorigenic fraction of cancer cells really composed only of cancer cells or could this fraction be highly contaminated by normal cells within the tumor? In such an event the depletion of tumorigenic activity in this fraction could be caused by the presence of normal cells rather than by the lack of tumorigenic activity in the cancer cells. Similarly, in tumors that contain large regions of necrotic tissue it is important to ensure that the nontumorigenic cancer cells are not simply cells that were fated to undergo cell death within the tumor. These alternative explanations require very careful experiments to evaluate. Nonetheless the reported evidence in support of the cancer stem cell model in testicular cancer, acute myeloid leukemia, breast cancer, colon cancer, and brain cancer makes these alternative explanations unlikely for those cancers.

Do Current Therapies Fail to Cure Cancer Because Cancer Stem Cells are Resistant?

Some have hypothesized that the failure of current therapies to cure disseminated solid malignancies is caused by the relative resistance of cancer stem cells to treatment. It is possible that in selecting therapies on the basis of their ability to shrink tumors, we have inadvertently selected therapies that preferentially kill the bulk population of nontumorigenic cancer cells (Figure 10-5). Therapies that specifically kill infrequent cancer stem cells might be more effective in curing disease but less effective in initially shrinking tumors (14). The hypothesis that current therapies are ineffective against cancer stem cells fits well with the clinical experience that therapies frequently shrink tumors to the point of undetectability but rarely cure metastatic disease. Perhaps cancer stem cells consistently survive current therapies and will continue to regenerate tumors unless they are surgically removed. Although this hypothesis seems compelling, it is important to remember that there is little experimental support for this hypothesis in the context of solid malignancies. It is possible that all solid cancer cells have the same sensitivity to therapy and that a similar fraction of cancer stem cells and nontumorigenic cancer cells survive therapy.

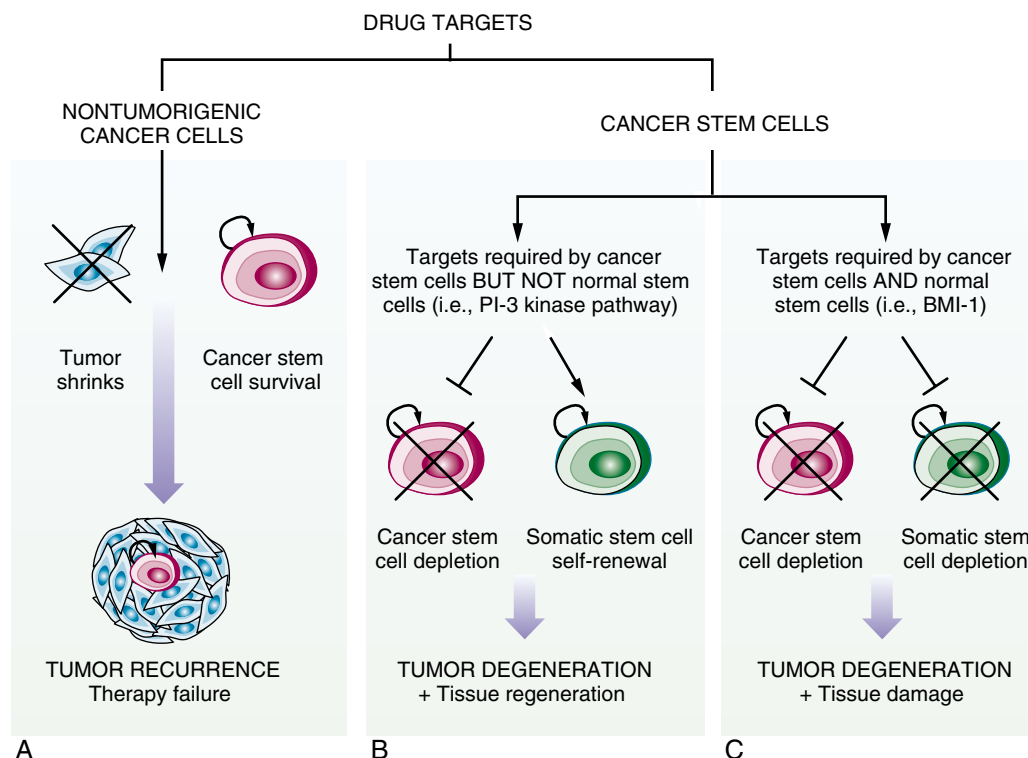


FIGURE 10-5 THERAPEUTIC IMPLICATIONS OF THE CANCER STEM CELL MODEL. There is concern that many anticancer therapies fail to cure metastatic disease because they preferentially eliminate the nontumorigenic cancer cells that compose the bulk of tumors (A). The failure to eliminate cancer stem cells would provide an opportunity for these cells to regenerate tumors, metastasize, or acquire additional mutations that confer drug resistance or more aggressive properties. Now that it is possible to identify cancer stem cells in certain cancers, it will be important to directly test the effectiveness of therapies against these cells. Therapies that directly target cancer stem cells can be thought to fall broadly into two classes. The first class comprise therapies that target cancer stem cells without harming the normal stem cells in the same tissue. Studies have identified drugs that can kill leukemic stem cells in various contexts without harming normal hematopoietic stem cells (B). The other class comprise therapies are generally toxic to both normal stem cells and cancer cells (C) or, in principle, could target pathways that are required by both normal stem cells and cancer stem cells, such as Bmi-1. If therapies can be identified that efficiently kill cancer stem cells without exhibiting intolerable toxicity to normal tissues, then such therapies could potentially cure metastatic disease regardless of whether nontumorigenic cancer cells are also eliminated. An example of a therapy that achieves this goal is the platinum compounds that are used to kill undifferentiated testicular cancer cells.

If so, the fundamental problem is the well-documented inability of therapies to kill every cancer cell, not biologic differences among cells in their sensitivity to therapy. It will be important to test these possibilities by directly comparing the effectiveness of therapies against solid cancer stem cells and other cancer cells.

There is some recent evidence that at least some types of solid cancer stem cells are more therapy resistant than other cancer cells from the same tumors. CD133⁺ glioma stem cells appear to be more resistant to radiation and more able to repair DNA damage than CD133⁻ glioma cells from the same tumors (104). CD133⁺ pancreatic cancer stem cells may also be more resistant to chemotherapy than CD133⁻ pancreatic cancer stem cells may also be more resistant to chemotherapy than CD133⁻ pancreatic cancer cells (105). Considerable additional work will be required to determine whether solid cancer stem cells are generally more therapy resistant than other cancer cells.

In the context of leukemia, there is evidence that leukemic stem cells are more resistant to some chemotherapies. Since leukemic stem cells are less mitotically active than other leukemic cells (41,42), leukemic stem cells would be expected to be less sensitive to chemotherapies designed to kill dividing cells. Indeed, chemotherapies that are used in the context of acute myeloid leukemia to target actively dividing cells, such as Ara-C and anthracyclines, are less toxic to leukemic stem cells than other leukemia cells in assays conducted in culture (65,66). Another potential example of

therapy resistance in cancer stem cells comes from chronic myeloid leukemia (CML) in which the BCR-ABL tyrosine kinase inhibitor imatinib mesylate (Gleevec, STI571; Novartis) has been remarkably effective in eradicating BCR-ABL⁺ chronic myeloid leukemia cells (67–69). Despite imatinib's effectiveness in patients, analysis of CD34⁺ progenitor cells from the bone marrow of patients before treatment revealed the presence of nondividing CD34⁺ cells, which were carrying the BCR-ABL mutation but were insensitive to imatinib in culture (70). Analysis of CML patients in remission after imatinib treatment revealed the presence of residual CD34⁺ BCR-ABL⁺ cells in the blood, which persisted even after continued imatinib treatment (71). Imatinib appears to kill dividing leukemic cells and to inhibit the proliferation of quiescent leukemic stem cells (70), but these quiescent cells can persist and acquire additional mutations, such as BCR-ABL amplification (72), leading to disease relapse (73). These observations suggest that therapy resistance in cancer stem cells can ultimately lead to therapy failure.

How Can We Kill Cancer Stem Cells?

As discussed previously, there are still many unknowns related to the nature and clinical significance of cancer stem cells. Even in cancers in which cancer stem cells have been demonstrated to exist, it is not clear

whether these cancers could be cured by therapies that specifically target cancer stem cells. Nonetheless, even if cancer stem cells are not the whole story, it is reasonable to expect that therapies that do a better job of killing cancer stem cells might achieve better results in patients. So how can we do a better job of killing cancer stem cells (Figure 10-5)?

Cancer stem cells are thought to use many of the same molecular mechanisms as normal stem cells to regulate their maintenance (13,14,46). For example, the Wnt, Sonic hedgehog, and Notch pathways that often promote cancer cell proliferation also promote normal stem cell self-renewal (13,14,46,74–76). Even as we discover new regulators of stem cell self-renewal, these regulators consistently play similar roles in positively or negatively regulating the self-renewal of normal stem cells and cancer stem cells. In addition to the common dependence on Bmi-1 exhibited by normal hematopoietic stem cells and leukemic stem cells (see acute myeloid leukemia section), Bmi-1 is also likely to be required for the maintenance of brain cancer stem cells (77), much as it is required for the maintenance of normal adult neural stem cells (50,52,78). The extensive mechanistic similarities between normal stem cell self-renewal and cancer stem cell self-renewal make it difficult to identify targets that can be exploited to kill cancer stem cells without damaging normal stem cells.

Research results demonstrate that it is possible to identify mechanistic differences between cancer stem cells and normal stem cells in the same tissue, and to target these differences to kill cancer stem cells without harming the normal stem cells. The lipid phosphatase, Pten (phosphatase and tensin homologue), is a tumor suppressor that negatively regulates cellular proliferation and survival by reducing signaling through the PI-3 kinase pathway. *Pten* is commonly deleted or inactivated in diverse cancers (79) including hematopoietic malignancies (80–83). Increased PI-3 kinase signaling in the absence of *Pten* leads to hyperactivation of the downstream kinases Akt and mTOR (mammalian target of rapamycin), which promote cellular proliferation and survival. Unlike most proto-oncogenes or tumor suppressors that have similar effects on normal stem cells and cancer cells, conditional deletion of *Pten* from adult mouse hematopoietic cells had opposite effects on hematopoietic stem cells and leukemic stem cells. *Pten* deletion caused the generation and expansion of transplantable leukemic stem cells, while leading to the depletion of normal hematopoietic stem cells (63). This identified a rare pathway that had opposite effects on cancer stem cells and normal stem cells.

To test whether this difference could be exploited to eliminate cancer stem cells without harming normal hematopoietic stem cells, rapamycin was administered to these mice (Figure 10-6). Rapamycin inhibits mTOR kinase activity, attenuating the increased signaling through the PI-3 kinase pathway that occurs in the absence of *Pten*. Rapamycin not only eliminated leukemic stem cells and restored the health of *Pten*-deficient mice, but it actually rescued the activity of *Pten*-deficient hematopoietic stem cells (63). These data demonstrate that it is possible to identify therapies that kill cancer stem cells without harming the normal stem cells in the same tissue. This raises the possibility that rapamycin analogues might be used along with other therapies in patients to eradicate cancer stem cells that depend on increased signaling through the PI-3 kinase pathway. Rapamycin is effective in killing clonogenic human leukemia cells (84,85)⁵ and preliminary data suggest that it can provide

some benefit when administered to patients with acute myeloid leukemia (86). However, it has been disappointing in other contexts when administered as a single agent (87,88), and therefore might need to be used in the context of minimal residual disease, or in combination with other therapies, where it could contribute to the elimination of residual cancer stem cells.

Other studies have also compared the sensitivity of leukemic stem cells and normal hematopoietic stem cells to chemotherapy. NF- κ B is constitutively activated in primitive acute myeloid leukemia cells but not in normal hematopoietic progenitors (66). Inhibition of NF- κ B using a proteasome inhibitor (MG-132) or the naturally occurring small molecule parthenolide induces apoptosis in cultured leukemic stem cells but spares normal hematopoietic stem cells (66,89). By combining the MG-132 proteasome inhibitor with the anthracycline, idarubicin, extensive p53-mediated cell death was induced in cultured leukemic stem cells while sparing normal hematopoietic stem cells (90). Importantly, the effects of these drug treatments on leukemic stem cells and normal hematopoietic stem cells were tested by transplantation of the cultured normal or leukemic stem cells into NOD/SCID mice to test their ability to give rise to normal hematopoiesis or leukemia, respectively. These studies demonstrated based on robust functional assays that certain chemotherapies can dramatically reduce the ability of leukemic stem cells to initiate leukemias in vivo without substantially damaging the ability of normal hematopoietic stem cells to engraft.

Another strategy to eradicate cancer stem cells and prevent the recurrence of tumors would be to induce the differentiation of cancer stem cells into nontumorigenic cancer cells. This approach effectively converts malignant cells into benign cells by inducing epigenetic changes that eliminate the clonogenic potential of the cancer stem cells (14). The possibility for “differentiation therapies” has been discussed in the literature ever since the cancer stem cell model was first proposed in the 1960s (91). Proof-of-principle for this approach comes from the effectiveness of all-*trans* retinoic acid as a therapy for acute promyelocytic leukemia (92–94). All-*trans* retinoic acid induces the terminal differentiation and apoptosis of the leukemic cells. An analogous approach has been used in the context of brain tumors in which treatment with bone morphogenetic proteins promoted the differentiation of human glioblastoma stem cells, reducing tumor growth in vivo (106). This general approach is likely to work in other contexts as well, as suggested by the observation that transient inactivation of the Myc oncogene in Myc-driven sarcoma cells leads to the differentiation of sarcoma cells into mature osteocytes (95). This differentiation is not reversed by reactivation of Myc, and the tumor does not recur. This demonstrates that even a transient loss of the signals that maintain the undifferentiated state of cancer cells can lead to an irreversible loss of proliferative potential.

Conclusions and Future Directions

By comparing the tumorigenic capacity of phenotypically distinct cancer cells from within individual tumors, it has become clear that some cancer cells are much more clonogenic than others. This is certainly true in testicular cancer, acute myeloid leukemia, brain cancer, colon cancer, and breast cancer. However, it remains

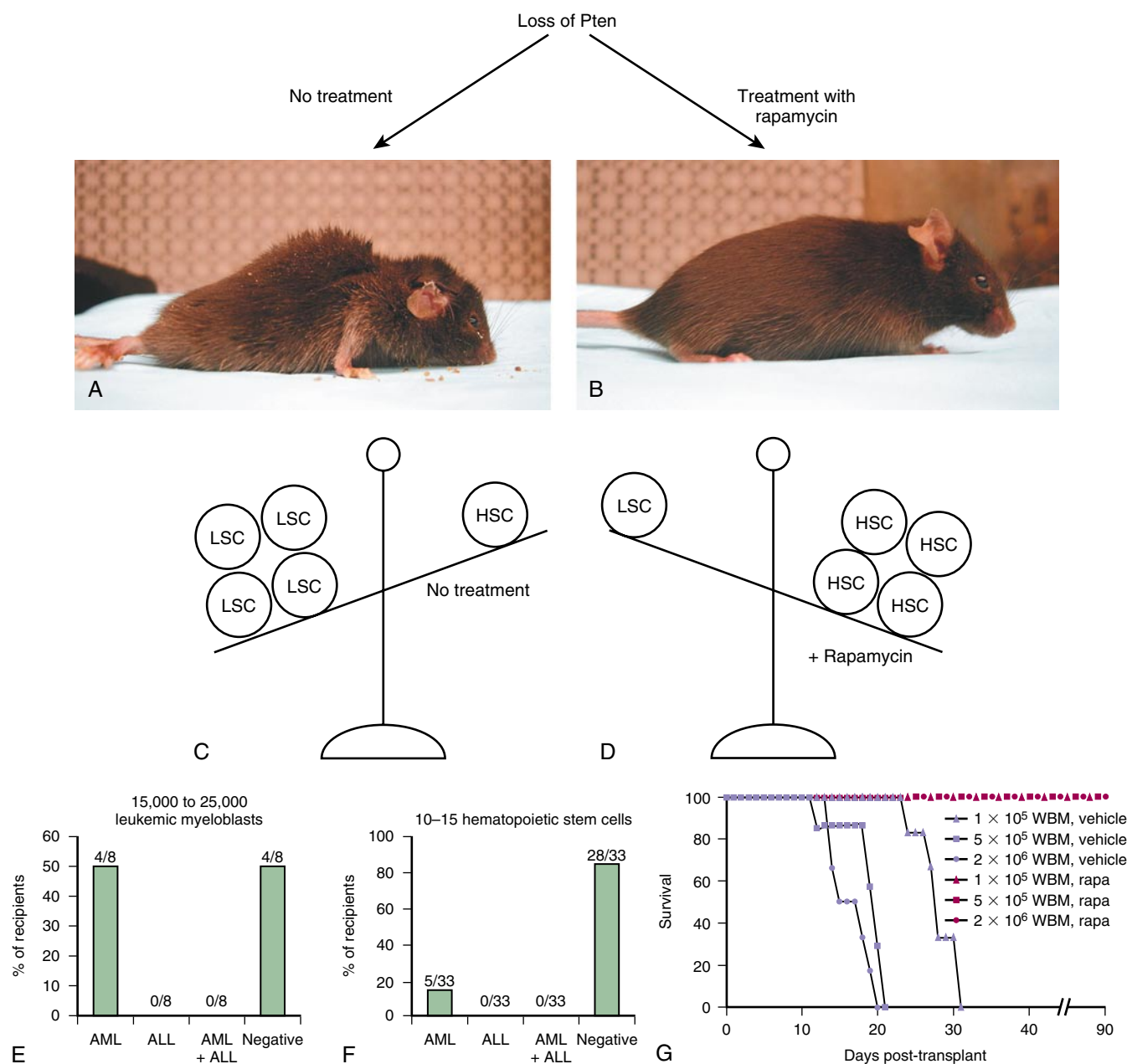


FIGURE 10-6 RAPAMYCIN ELIMINATES LEUKEMIC STEM CELLS WITHOUT HARMING NORMAL HEMATOPOIETIC STEM CELLS. Conditional deletion of the *Pten* tumor suppressor gene from mouse hematopoietic cells leads to the rapid onset of acute myeloid leukemia and acute lymphoblastic leukemia, typically leading to the death of mice within 6 weeks of *Pten* deletion (A). The leukemias that arise in this mouse follow a cancer stem cell model in which cells that express hematopoietic stem cell markers are greatly enriched for the ability to initiate secondary leukemias (F) as compared with leukemic blast cells (E). Thus *Pten* deletion leads to the generation and maintenance of leukemic stem cells (LSCs; C). In parallel, *Pten* deficiency cell autonomously leads to the depletion of normal hematopoietic stem cells (C). *Pten* deletion therefore identifies a rare mechanistic distinction between cancer stem cells and normal stem cells. Targeting the phosphatidylinositol-3-kinase (PI3K) pathway with rapamycin to reduce pathway activation downstream of *Pten* eliminated leukemic stem cells while rescuing normal hematopoietic stem cell function (D) and restoring the health of mice (B). Transplantation assays confirmed that whole bone marrow (WBM) from vehicle-treated mice killed mice on transplantation in a dose-dependent manner, whereas WBM from rapamycin-treated mice never transferred disease (G). This and other work proves that it is possible to identify mechanistic differences between leukemic stem cells and normal hematopoietic stem cells that can be targeted to eliminate the leukemic stem cells without harming the normal stem cells. (From Yilmaz OH, Valdez R, Theisen BK, et al. *Pten* dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 2006;441:475, with permission.)

to be determined whether this will be true in most or all cancers or whether there will be significant numbers of cancers in which most cancer cells have a similar capacity to form new tumors. For now, it remains critical to rigorously test the cancer stem cell model in each cancer before embarking on therapeutic or experimental approaches that assume its validity. Even among the cancers in

which the model has been proven, it remains possible that there will be subtypes of leukemia, breast cancer, or brain cancer in which the model does not hold up.

The cancer stem cell model is consistent with a role for ongoing mutagenesis in influencing the growth and progression of cancer. The cancer stem cell model simply contributes an additional

insight: There are also epigenetic differences among cancer cells that collaborate with ongoing genetic change to generate heterogeneity. It is thus not necessary to attribute all phenotypic and functional differences among cancer cells to genetic change. Genomic instability and ongoing mutations likely change the properties of cancer stem cells over time, and presumably are responsible for the acquisition of drug resistance by these cells.

The cancer stem cell model is also consistent with the idea that oncogenic mutations often act by causing differentiation arrest (34). Indeed, cancer stem cells may self-renew in a dysregulated manner precisely because of mutations that inactivate normal differentiation pathways in some cases. For example, it has been proposed that constitutive activation of β -catenin in restricted myeloid progenitors impairs their maturation into fully differentiated myeloid cells and confers on these cells increased self-renewal potential and prolonged survival, leading to chronic myeloid leukemia (16). Obviously differentiation arrest is neither complete nor certain, as cancers such as teratocarcinoma show abundant and diverse differentiation to postmitotic cells. Moreover, even in cases in which the inactivation of differentiation pathways contributes to neoplastic proliferation, epigenetic changes could still reduce proliferative potential without being associated with overt or recognizable differentiation. For example, breast cancer stem cells would appear to undergo epigenetic changes that reduce their tumorigenic capacity as they give rise to nontumorigenic breast cancer cells despite the fact that these nontumorigenic cells often do not show obvious signs of differentiation. These observations indicate that reduced proliferative capacity can be uncoupled from the expression of differentiation markers, and that "left-shifted" neoplasms marked by the expansion of immature cells can still be composed of cells with heterogeneous proliferative potentials. A critical question for the field is what is the nature of the epigenetic changes that distinguish cancer stem cells from their undifferentiated but nontumorigenic progeny.

Another obvious prediction from the cancer stem cell model is that metastases are derived from the dissemination of cancer stem cells and not from the dissemination of nontumorigenic cancer cells. This idea is attractive because it has long been observed that circulating cancer cells can be detected in patients who never develop metastatic disease (25,96). Of course, it remains possible that most circulating cancer cells are eliminated by immune surveillance. However, if cancer stem cells are uniquely tumorigenic among cancer cells, it follows that only these cells should be able to spread systemic disease (97,105). Although attractive, it is important to remember that this is only a hypothesis at present, with little direct experimental support. Nonetheless, this raises the possibility that cancer stem cells may exhibit intrinsic differences in migratory properties and metastatic potential relative to other cancer cells, in addition to their demonstrated differences in proliferative potential.

In addition to the obvious possibilities regarding new therapeutic approaches, the identification of cancer stem cells also raises new possibilities for diagnosis. For many cancers the prognosis depends on early detection. Yet detection often means discerning palpable or radiologically evident masses of abnormal cells. The identification of cancer stem cells based on their expression of unique combinations of surface markers raises the possibility of achieving single-cell assays for the presence of malignant cells. To be sure, we are not there yet. The surface markers that have been used to isolate cancer stem cells from the blood, breast, colon, and brain may distinguish these cells from other tumor cells, but they do not clearly distinguish these cells from normal stem cells in those tissues. Additional work is required to develop single-cell assays that can detect cancer stem cells based on phenotype or function. Such assays could potentially be applied to blood, breast ductal lavage, lung lavage, or other sources of cells used for screening. Such an approach could revolutionize cancer diagnosis in a way that could have an even greater impact on cancer survival than new therapies to target cancer stem cells.

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Signal Transduction by Growth Factor Receptors

Signaling: Components and Devices

The behavior of a cell in the human body is shaped first by its developmental history during embryogenesis, which determines the repertoire of gene products that it expresses and thus the range of its biologic properties, as well as its spatial integration into a particular tissue. In the adult, cells can also undergo rapid changes, as occurs in response to infection or wounding, or during normal hematopoiesis. During embryonic development and in postnatal tissues, the function of an individual cell depends on communication with its environment. A cell's most immediate contacts are with the extracellular matrix and adjacent cells, and these are essential for maintaining its architecture and functional properties. For example, the interactions of an epithelial cell with its neighbors, and with the underlying matrix, are crucial for the formation of specialized junctions between cells and for the polarized distribution of macromolecules within a single cell. These features, in turn, are essential in maintaining the integrity of epithelial cell layers that line organs such as the intestine or the lung. The division of an epithelial cell into two daughter cells must also be highly organized in space, since this will determine whether the new cells remain in the epithelium or can leave the epithelial monolayer to adopt a new fate. Local effects can be achieved through the direct interaction of two proteins anchored to the surface of adjacent cells (e.g., two cadherin molecules undergoing a homotypic interaction) or through the association of a cell surface protein with a component of the extracellular matrix (e.g., an integrin heterodimer binding to fibronectin or laminin). Alternatively, one cell may secrete a soluble growth factor (often a polypeptide hormone), which binds to the extracellular region of a transmembrane receptor of a nearby target cell, which responds by undergoing specific phenotypic alterations. An example of this latter scheme is the production of platelet-derived growth factors (PDGFs) to stimulate the expansion of mesenchymal cells in the vasculature (1). In addition to cues from their immediate environment, cells respond continuously to signals that emanate from distant sites in the body, notably endocrine organs that release hormones such as insulin into the circulation.

Signal transduction describes the process through which extracellular signaling molecules bind specific receptors on target cells, which consequently activate selected intracellular biochemical pathways. These control facets of cellular organization such

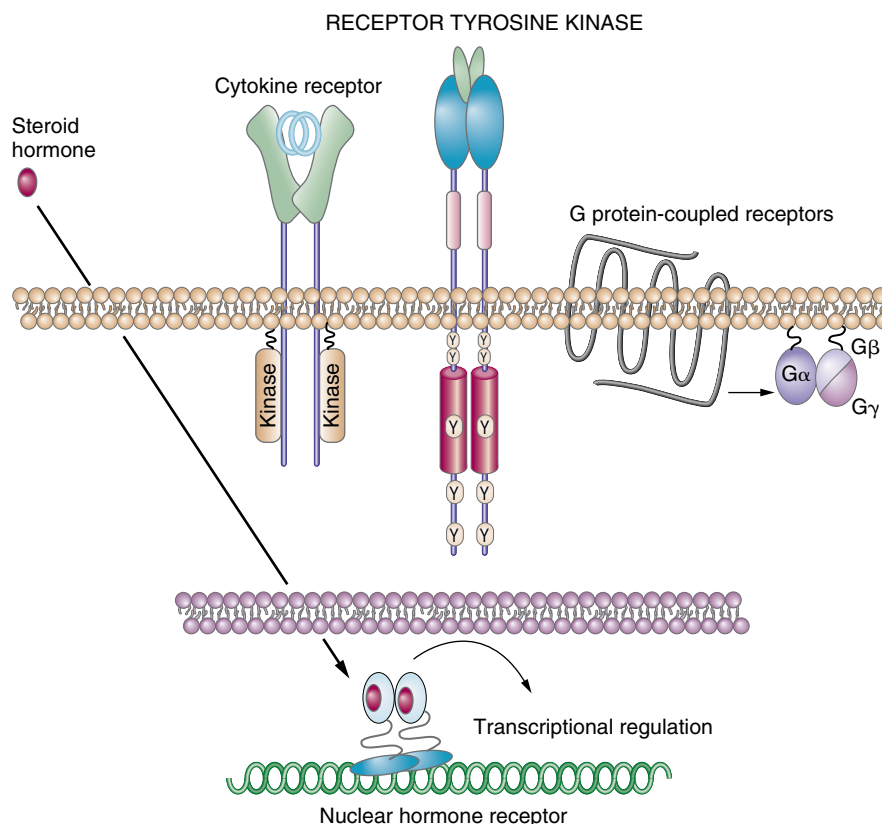
as gene expression, cytoskeletal architecture and motility, growth, division, survival, metabolism, and differentiation. As a simplifying principle, the cell can be viewed as containing core machines in the form of macromolecular assemblies that operate these various cellular activities. In the course of evolution, signaling pathways have become overlaid on these core machines, so that they become responsive to the specialized external environment of a cell in a multicellular animal. Since at any one time a cell is likely to be exposed to numerous different signals, it must have mechanisms to integrate such stimuli into a coherent response. The cell must also be able to rapidly attenuate signals that would be dangerous if left unchecked, and make choices on the basis of the strength of a given cue. For example, the affinity with which an antigen binds an antigen receptor on lymphoid cells determines whether the cell responds by undergoing death, quiescence, or proliferation. Not surprisingly, aberrations in signal transduction pathways represent a central mechanism that drives the growth of tumors and are therefore a promising source of targets for the new generation of anticancer reagents.

In detail, signaling pathways can become extremely complex, but they nonetheless are built from a rather limited tool kit of molecular devices. We will first describe some underlying properties of signaling molecules and then discuss their use in particular pathways that transmit information from the cell surface to internal targets.

Receptors

Most extracellular signaling molecules do not readily enter the cell, and the proteins which act as their receptors must therefore traverse the plasma membrane (Figure 11-1). Receptors for growth factors, cytokines, antigens, and guidance molecules typically have an N-terminal extracellular region that binds selectively and with high affinity to the appropriate ligand, a single-membrane-spanning segment, and a cytoplasmic region that engages intracellular targets once the receptor is activated by its physiological ligand (2). Typically, activation of such receptors is accomplished by clustering of individual polypeptide chains into dimers or oligomers (3). Most receptors discussed in the following sections, for example, receptor tyrosine kinases (RTKs), fall into this group. A distinct class of receptors, which have a wide range of protein

FIGURE 11-1 CELLS POSSESS DIVERSE RECEPTORS FOR EXTRACELLULAR SIGNALS. Initiation of signal transduction through the interaction of an extracellular ligand with a specific receptor can take several forms. Steroid hormones readily cross cellular membranes and can therefore bind directly to intracellular nuclear hormone receptors, which regulate gene expression. Polypeptide growth factors and cytokines, in contrast, bind the extracellular regions of transmembrane receptors with intrinsic or associated tyrosine kinase activity. A G-protein-coupled receptor is shown for comparison.



and nonprotein extracellular ligands, possesses seven transmembrane segments and undergo a conformational change upon ligand-binding, which stimulates their ability to activate heterotrimeric G-proteins within the cell. These receptors are commonly termed “G-protein-coupled receptors” (GPCRs; 4). In contrast, signaling molecules such as steroids can directly penetrate the plasma membrane, and consequently bind receptors (nuclear hormone receptors) that are entirely intracellular; these receptors commonly act directly as transcription factors, whose ability to regulate gene expression is controlled by ligand-binding (5,6).

Phosphorylation

Rapid alterations in cellular state are frequently achieved by post-translational modifications of existing proteins, for which phosphorylation on the hydroxyamino acids serine, threonine, and tyrosine is the prototypic example (Figure 11-2A). Protein phosphorylation is mediated by protein kinases, of which there are at least 518 encoded by the human genome (7) and reversed by specific phosphatases (8). Phosphorylation can alter the function of a protein in two general ways: (1) by inducing new molecular contacts within the phosphorylated protein such that it adopts a new structural conformation, with altered biochemical properties (9,10) or (2) by creating binding sites for protein domains that selectively recognize phosphorylated motifs, resulting in a novel protein-protein interaction (11–13). Phosphorylation is one of many post-translational modifications, which include acetylation (for example on lysine), methylation (i.e., on lysine or arginine), prolyl hydroxylation, or ubiquitylation on lysine residues (see following sections; 14,15).

These modifications can act in combination; for example the same protein can be phosphorylated at multiple sites, or both phosphorylated and ubiquitylated. Consequently, post-translational modifications provide a versatile mechanism by which a protein can rapidly be converted to one of several new functional states.

Protein-Protein and Protein-Phospholipid Interactions

Most human proteins are modular, in the sense that they are composed of multiple structurally independent domains that possess catalytic activity (as in the case of protein kinase domains) or mediate specific molecular interactions (Figures 11-2A and 11-2B; 15,16). Interaction domains frequently recognize short peptide sequences in their binding partners, and in some cases, this association is dependent on ligand phosphorylation. For example, Src homology-2 (SH2) domains (of which there are 120 in the human proteome) have a conserved phosphotyrosine (pTyr)-binding pocket and thus bind specifically to peptide motifs that have been phosphorylated on tyrosine residues (17,18). In addition to their conserved ability to bind pTyr, SH2 domains recognize amino acids N- and C-terminal to the phosphorylated site, in a fashion that varies from one SH2 domain to another, imparting a degree of selectivity in SH2 domain-mediated interactions (19,20). There are additional domain families (e.g., PTB domains) with a propensity to bind pTyr-containing motifs, and several families of interaction domains or full-length proteins that specifically recognize sites of serine/threonine phosphorylation (e.g., 14-3-3 proteins discussed in subsequent sections; 12,21). In this fashion,

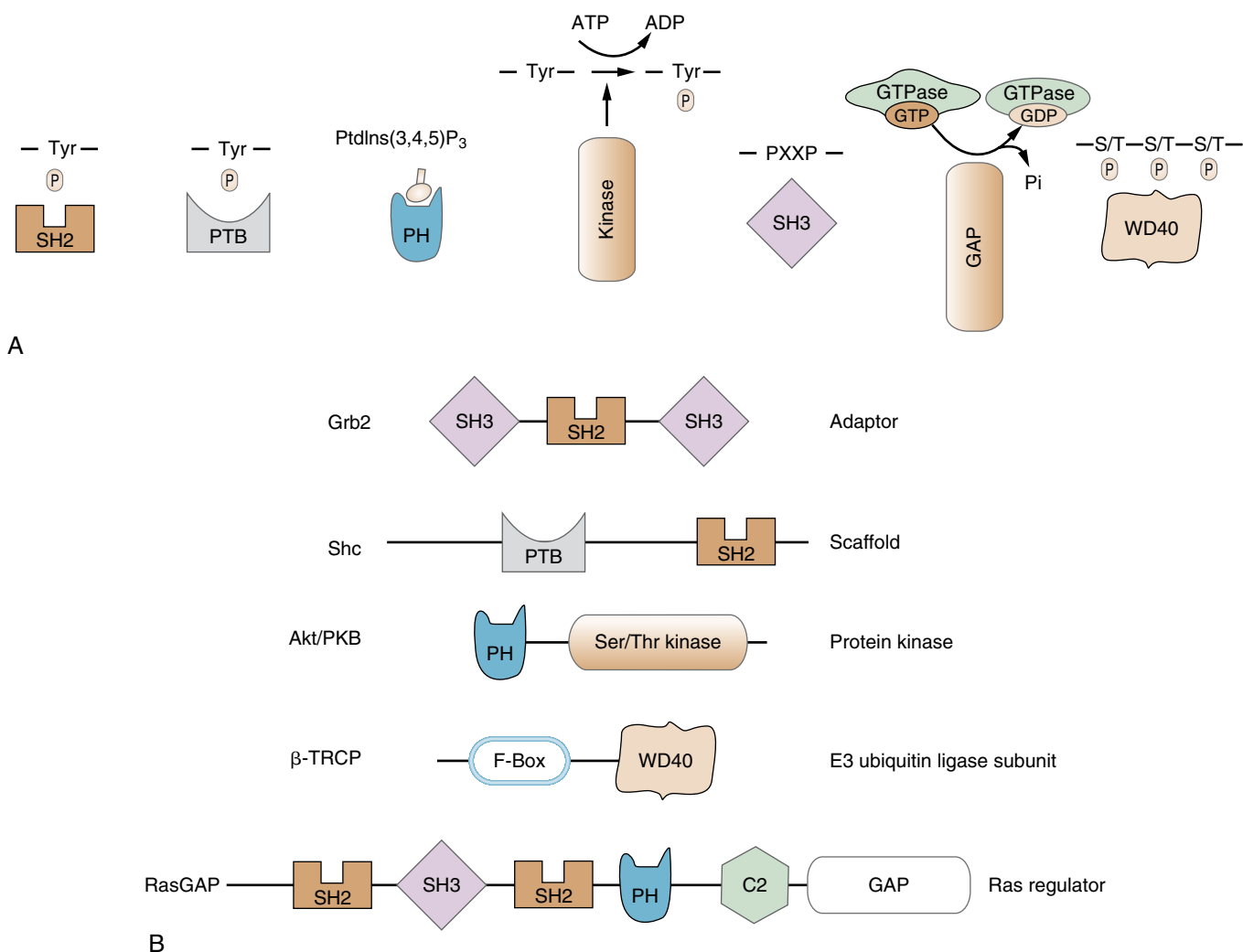


FIGURE 11-2 MODULAR DEVICES AND THEIR FUNCTIONS. Protein modules serve to coordinate signaling complexes by specifically recognizing appropriate ligands or substrates. **A:** Selected interaction and catalytic domains and their cognate binding motifs or substrates. **B:** Five proteins are depicted that reflect the multidomain nature of signaling polypeptides.

protein phosphorylation can drive the formation of specific, multiprotein complexes, capable of transmitting a downstream signal within the cell. Interaction domains can also associate with other types of peptide motifs, as in the case of SH3 domains that bind proline-rich sequences (Figure 11-2A; 22). Alternatively, they bind other folded domains, typified by death domains, which form heterodimers in signaling from receptors that induce apoptosis (such as the Fas receptor; 23).

Interaction domains are not restricted to the recognition of peptide ligands, but can also bind phospholipids, such as phosphoinositides (24,25) as well as nucleic acids, small molecules, and metabolites. For example, some PH domains (Figure 11-2A) selectively recognize phosphatidylinositol(4,5) P_2 (PI(4,5) P_2), in which phosphate has been incorporated into the D4 and D5 positions of the inositol ring in the phospholipid head group, whereas other PH domains bind PI(3,4,5) P_3 , which is generated from PI(4,5) P_2 by the action of a phospholipid kinase, PI3K, phosphatidylinositol-3-kinase (PI3K; 26,27). Because these phospholipids are embedded in the plasma membrane, their formation can recruit proteins with the appropriate PH domains to specific membrane sites.

Adaptors and Scaffolds

A critical aspect of intracellular signaling involves the physical recruitment of proteins that lie on the same pathway into common multiprotein complexes. This is often achieved by adaptor proteins, composed of several distinct interaction domains (28–30). Adaptors can selectively bind to activated receptors and to cytoplasmic targets that regulate intracellular signaling pathways. For example, adaptors with SH2 and SH3 domains such as Grb2 (Figures 11-2B and 11-5; discussed in subsequent sections) bind pTyr-containing sites in activated RTKs through their SH2 domain and proline-rich motifs in cytoplasmic effectors through their SH3 domains. Similarly, scaffold proteins can recruit multiple components of a signaling pathway, as seen for scaffolds that bind successive protein kinases in MAP kinase pathways, and thereby enhance the specificity with which a signal is transmitted (Figure 11-4; 31). Scaffolds can also exert sophisticated functions that control the extent and duration of intracellular signaling. For example, a class of scaffolds known as A-kinase-anchoring proteins (AKAPs) binds the cyclic adenosine monophosphate

(cAMP)–dependent protein kinase A (PKA) in an inactive state and tethers this enzyme close to its substrates, which become phosphorylated by the catalytic subunit when this is released by elevated cAMP (32). However, AKAPs also bind protein phosphatases, as well as phosphodiesterases that degrade cAMP, and can thereby rapidly attenuate PKA-mediated phosphorylation (33). Adaptors and scaffolds can therefore play important roles in determining which signaling pathways are activated by receptors, where in the cell signaling complexes form, and the duration of signaling events.

GTPases

GTPases, such as Ras proteins, toggle between inactive and active conformations, based on their binding to the guanine nucleotides GDP or GTP (Figure 11-3; 34–36). GTPases are in the “OFF” state when bound to GDP and are activated by guanine nucleotide exchange factors (GEFs), which open up the protein and cause GDP to be released. Because the concentration of GTP in the cell is much higher than GDP, the GTPase consequently associates with GTP and undergoes a structural change. In this new GTP-bound “ON” state the GTPase binds and activates a number of downstream targets with appropriate GTPase-binding domains. Although they are called “GTPases,” the intrinsic GTPase activity of Ras-like proteins is rather weak, but this is stimulated by separate GTPase activating proteins (GAPs), which consequently help to hydrolyze bound GTP back to GDP, and thereby turn the GTPase off. Thus GTPases are switch-like proteins that are activated to bind their targets by GEFs, and are then shut off by GAPs. Ras proteins are associated with the plasma membrane (see subsequent section), and can activate downstream effectors by inducing their recruitment to the membrane, and by disrupting their intramolecular autoinhibitory interactions (37).

Activated GPCRs function as their own GEFs, but rather than stimulating Ras-like GTPases, they induce the exchange of GDP for GTP on $G\alpha$ proteins, which consequently dissociate from the $G\beta$ and $G\gamma$ subunits with which they interact when in the GDP-bound state (38). Both GTP-bound $G\alpha$ proteins, as well as the released $G\beta/\gamma$ heterodimer, can interact with downstream effectors (39).

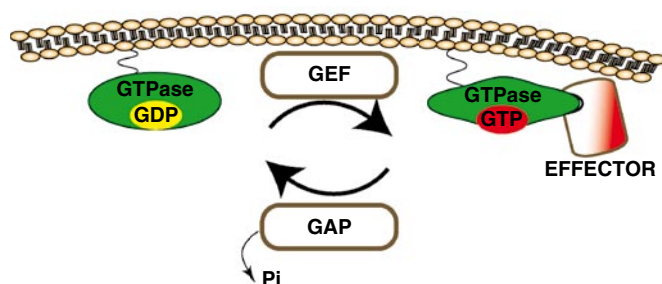


FIGURE 11-3 THE GTPASE CYCLE. Small GTPases toggle between their GDP (inactive) and GTP (active) bound forms. Activated guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, inducing an activated conformation that binds downstream targets with an appropriate Ras-binding domain. In contrast, GTPase-activating proteins (GAPs) stimulate GTP hydrolysis, leading to inactivation.

Proteolysis and Ubiquitination

Targeted proteolysis and protein degradation can play pivotal roles in signaling pathways. Proteins are marked for destruction by their attachment to ubiquitin, a 76–amino acid peptide that is joined through a C-terminal glycine to lysine residues in the targeted protein, through the formation of an isopeptide bond (40,41). This type of post-translational modification can become quite complex, as ubiquitin itself has several lysine residues that can be ubiquitinated (i.e., Lys48, Lys63), leading to the formation of polyubiquitin chains. Ubiquitination is carried out by a series of enzymes (Figure 11-4A), including the E1 ubiquitin-activating enzyme, which becomes covalently linked to ubiquitin at an active site cysteine through a thioester bond, and an E2 ubiquitin-conjugating enzyme which accepts ubiquitin from the E1 protein by a transesterification reaction. E3 protein–ubiquitin ligases bind to the E2 and the protein substrate for ubiquitination, and thus recruit the target protein to the ubiquitinating machinery (42). E3 ligases of the HECT family can themselves form a covalent intermediate with ubiquitin before it is attached to the target, whereas other E3 proteins act purely as adaptors to juxtapose an E2 to its target, as in the case of E3 ligases with Ring finger domains (43,44). Interestingly, the substrate-binding domains of some E3 ligases only recognize their targets following phosphorylation of the target protein. For example, cytoplasmic β -catenin is phosphorylated on a DSGXXS motif by the GSK-3 protein kinase and is therefore recognized by the WD40 repeat domain of β -TRCP (Figures 11-2B and 11-4B), a component of a multisubunit E3 ligase complex; consequently β -catenin is polyubiquitinated and degraded (45). Signaling by secreted factor Wnt, acting through the Frizzled receptor, blocks β -catenin phosphorylation, resulting in its stabilization, retention in the nucleus, and regulation of gene expression in conjunction with Tcf transcription factors (46,47).

In the same way that pTyr sites are recognized by SH2 domains, ubiquitinated sites are recognized by proteins containing a variety of ubiquitin binding domains (UBDs; 48,49). By this mechanism, proteins that are polyubiquitinated through Lys48 linkages are recognized by the proteasome, and degraded. In some cases, however, E3 ligases add a single ubiquitin chain to a target lysine, which is therefore monoubiquitinated. For example, specific pTyr sites on activated RTKs can be recognized by the variant SH2 domain of a Cbl E3 ligase, which then monoubiquitinates the receptor and adds Lys63-linked polyubiquitin (50,51). These ubiquitinated sites are recognized by the UBDs of proteins involved in endocytosis, promoting receptor internalization and down-regulation (52).

Specific proteolysis that liberates a novel polypeptide fragment from a larger protein can also be an important device for promoting signaling. For example, binding of extracellular ligands to Notch receptors promotes their cleavage at specific sites, producing a cytoplasmic Notch fragment that moves to the nucleus to control gene expression (53).

A defining characteristic of proteolysis and protein degradation is that they are not immediately reversible, unlike phosphorylation, and can therefore be used to allow the cell to

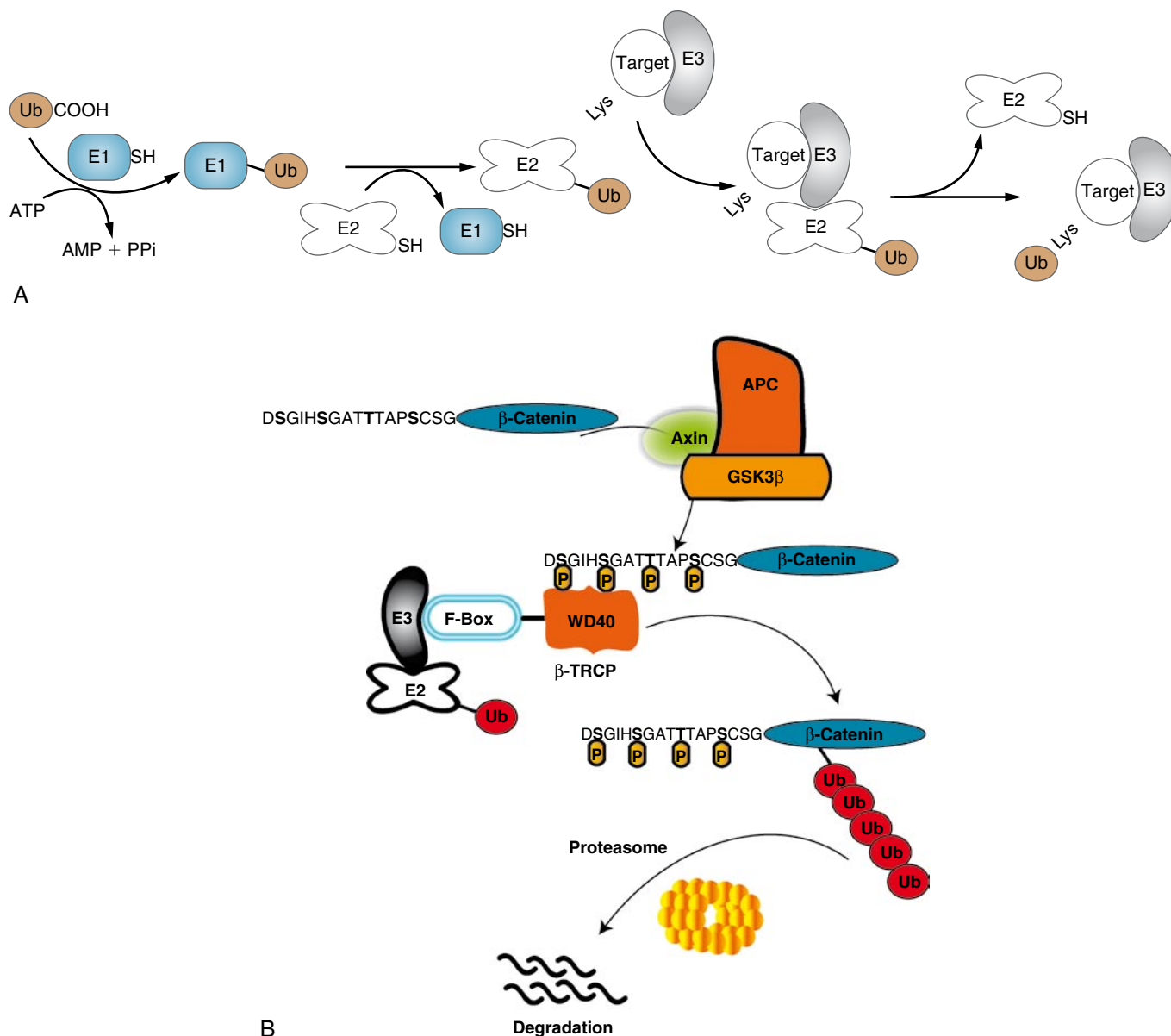


FIGURE 11-4 **UBIQUITYLATION AND DEGRADATION.** **A:** Schematic representation of the ubiquitylation pathway. Covalent attachment of ubiquitin to the E1 ubiquitin-activating enzyme is an adenosine triphosphate (ATP)-dependent reaction. From the E1 subunit, ubiquitin is then transferred to one of several different E2 ubiquitin-conjugating enzymes through a transthiesterification reaction. The E2-ubiquitin/E3 protein-ubiquitin ligases form a complex with the substrate and the activated ubiquitin is transferred to the substrate. **B:** Ubiquitylation and degradation of β -catenin. In unstimulated cells, free β -catenin is recognized and phosphorylated by an Axin/APC/GSK-3 β complex. This phosphorylation event creates a recognition site for the WD40 domain of the F-box protein β -TRCP. Targeting of β -catenin to the SCF β^{TRCP} results in its polyubiquitination and subsequent degradation by the 26S proteasome.

undergo significant transitions. This is particularly evident in cell division, where the degradation of proteins such as cyclins is used for passage through the various stages of the cell cycle (54).

Feedback and Cross-Talk

Signaling pathways that lie downstream of RTKs contain a variety of feedback loops that control the amplification and duration of the signal through the pathway. Feedback can have a positive effect, by further enhancing signaling by a preceding step, or can be inhibitory and act to shut the pathway down.

As an example of negative feedback, the p70 S6 protein kinase (S6K), which is targeted by the PI3K pathway downstream of the insulin receptor (see subsequent section), phosphorylates the IRS-1 docking protein. IRS-1 is itself a substrate for the insulin RTK, which recruits PI3K to the activated receptor (55). Its phosphorylation on serine/threonine by S6K inhibits IRS-1 function, in part by interfering with its ability to bind the insulin receptor, and consequently shuts down insulin receptor signaling (56–58).

Members of distinct pathways can also regulate one another, in a process termed cross-talk. The Ras GTPase, which

conventionally stimulates the Raf-MEK-ERK MAP kinase (MAPK) pathway (see subsequent sections) can also bind and activate PI3K (59). Furthermore, the ERK MAPK can phosphorylate and inhibit tuberlin (TSC2), a GAP for the Rheb GTPase, which acts as a negative regulator of signaling downstream of PI3K (60). The ERK MAPK can therefore augment the PI3K signaling pathway. Thus, although it is convenient to think in terms of linear signaling pathways, there are many molecular interconnections between pathways. These establish a more complex signaling network through which signals can potentially be propagated to influence multiple targets.

Disruption of Cell Signaling in Cancer

The underlying molecular mechanisms used in the assembly of normal signaling pathways show a number of common properties. In particular, they allow signaling proteins to undergo a switchlike activation from an inactive to an active state (for example by receptor clustering, GTP-binding to Ras proteins, stabilization of β -catenin), and can also be readily reversed (i.e., by receptor down-regulation, hydrolysis of bound GTP, β -catenin degradation). Oncogenic mutations in a signaling protein tend to promote formation of its active state, while suppressing its ability to be inactivated. Conversely, tumor suppressor mutations can inactivate the proteins that normally attenuate signaling proteins. Both types of mutation can lead to the constitutive activation of pathways that promote cell growth, proliferation, survival, and invasion.

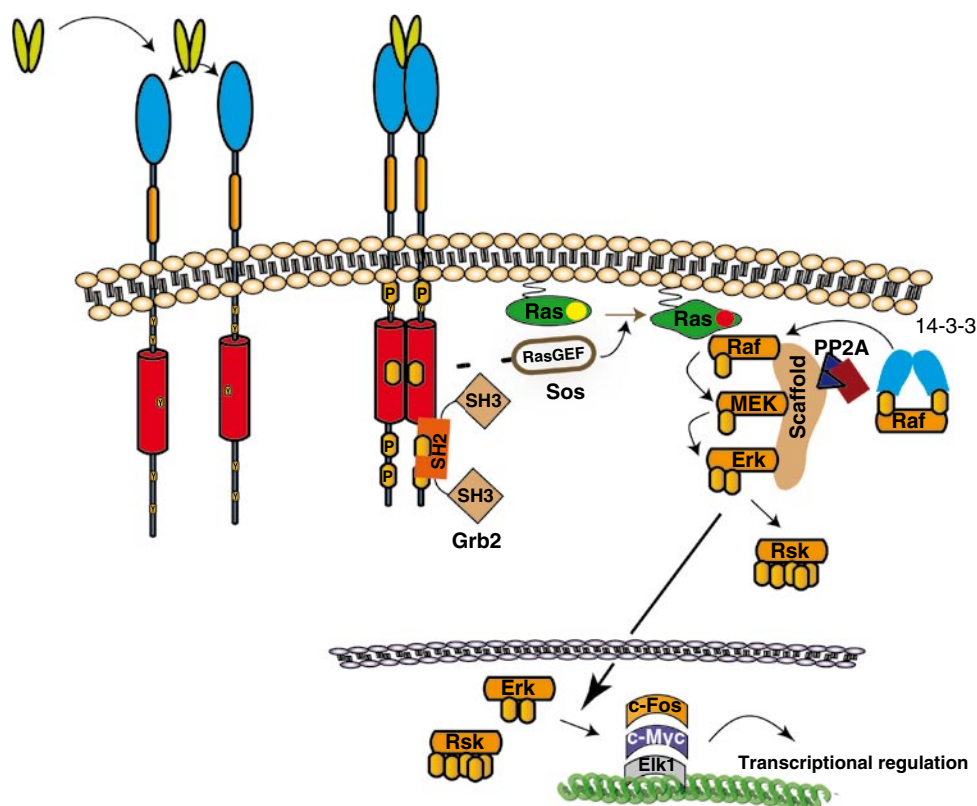
Signaling by Protein-Tyrosine Kinases

Activation of Growth Factor Receptors

Protein-tyrosine kinases are enzymes that transfer the γ -phosphate of ATP to the tyrosine residue of a substrate protein (61). If the protein that becomes phosphorylated is the kinase itself, which could occur by an intra- or intermolecular reaction, the process is termed "autophosphorylation." Tyrosine kinases can be divided into two groups: transmembrane receptors that directly bind extracellular ligands and intracellular cytoplasmic proteins (Figure 11-1). This latter class of cytoplasmic tyrosine kinases, which includes proteins such as Src, Abl, Btk, ZAP-70, JAK, and FAK, can act as the signaling subunits of multichain receptors (e.g., antigen or cytokine receptors or integrins), or serve as highly connected hub proteins in controlling signaling pathways and cytoskeletal architecture (62–67).

RTKs possess an extracellular ligand-binding region and a single transmembrane sequence that connects to a juxtamembrane region within the cytoplasm, followed by the kinase domain and a noncatalytic C-terminal tail (2). Human RTKs can be grouped into several families of closely related receptors, which correspond to their preference for similar ligands, such as epidermal growth factor (EGF), PDGF or fibroblast growth factor (FGF) family members (9). The binding of RTKs to their extracellular ligands commonly induces receptor dimerization (Figure 11-5), and this can be achieved in a number of ways. Most simply, growth factors such as PDGF form a covalent dimer, which directly contacts

FIGURE 11-5 ACTIVATION OF THE RAS/RAF/MAPK PATHWAY BY RECEPTOR TYROSINE KINASES. Receptor tyrosine kinases are activated by dimerization and resulting intermolecular autophosphorylation. Phosphorylation of tyrosine residues creates selective binding sites for the SH2 domains of intracellular targets. One of these is the adaptor protein Grb2, which recruits Sos, a Ras-GEF, through binding of the Grb2 SH3 domains to proline-rich motifs on Sos. The GTP-bound Ras activates the downstream MAPK pathway consisting of a MAPKKK (Raf), a MAPKK (MEK), and two MAPKs (Erk1/2). Activated Erk1/2 phosphorylates both cytoplasmic and nuclear targets.



and juxtaposes two receptor chains (1). In contrast, one molecule of EGF binds a single receptor chain at a site distant from the receptor dimerization interface. However, EGF binding stabilizes a conformation of the EGF receptor in which a dimerization arm is extended toward its partner, allowing two receptor chains to engage one another (68,69). The EGF receptor belongs to the ErbB family, which has four members capable of homo- or heterodimerization. Each receptor heterodimer can respond to a distinct set of extracellular ligands and has different intracellular signaling properties (70). Intriguingly, the ErbB2 receptor (known as HER2/neu) lacks intrinsic growth factor-binding activity and has its dimerization arm in a constitutively extended position. As a consequence, in normal cells ErbB2 must function as part of a heterodimer with another ErbB family member. ErbB2 expression is amplified in some cancers, notably a subset of breast cancers that are consequently sensitive to treatment with the therapeutic anti-ErbB2 antibody Herceptin (trastuzumab; 71,72). Conversely, ErbB3 can bind growth factors, but its kinase domain lacks catalytic activity, and it therefore serves as a scaffold for the recruitment of cytoplasmic targets following its phosphorylation by a heterodimeric partner, such as ErbB2. ErbB3 has a cluster of binding sites for PI3K (see subsequent sections), and heterodimers containing an ErbB3 chain will therefore have a propensity to strongly activate the PI3K

pathway. These data show that the simple mechanism of receptor dimerization is exploited in human cells to generate considerable biologic diversity, both in responsiveness to growth factors and in the activation of intracellular signaling pathways.

A consequence of receptor dimerization is that the kinase domain of one receptor chain is positioned so that it can phosphorylate its neighbor, resulting in a mutual intermolecular autophosphorylation. This autophosphorylation has two consequences, one being to stimulate the activity of the receptor, and the other to create docking sites for proteins with SH2 or PTB domains (Figures 11-2, 11-5, and 11-6; 9,20). Protein kinase domains have an N-terminal lobe and a larger C-terminal lobe. The active site, formed by the ATP-binding pocket and residues that mediate phosphotransfer, is located at the interface between the two lobes. In the inactive state, a sequence within the large lobe termed the “activation segment” typically occludes the active site (9,73). Autophosphorylation at one or more tyrosine residues in the activation segment results in a conformational change that moves this region away from the active site, thereby promoting catalytic activity. The EGF receptor, in contrast, may be catalytically activated by contacts made between the large lobe of one kinase domain with the small lobe of its neighbor, which consequently adopts an active conformation (74). In all such models, dimerization of the receptor allows two juxtaposed kinase

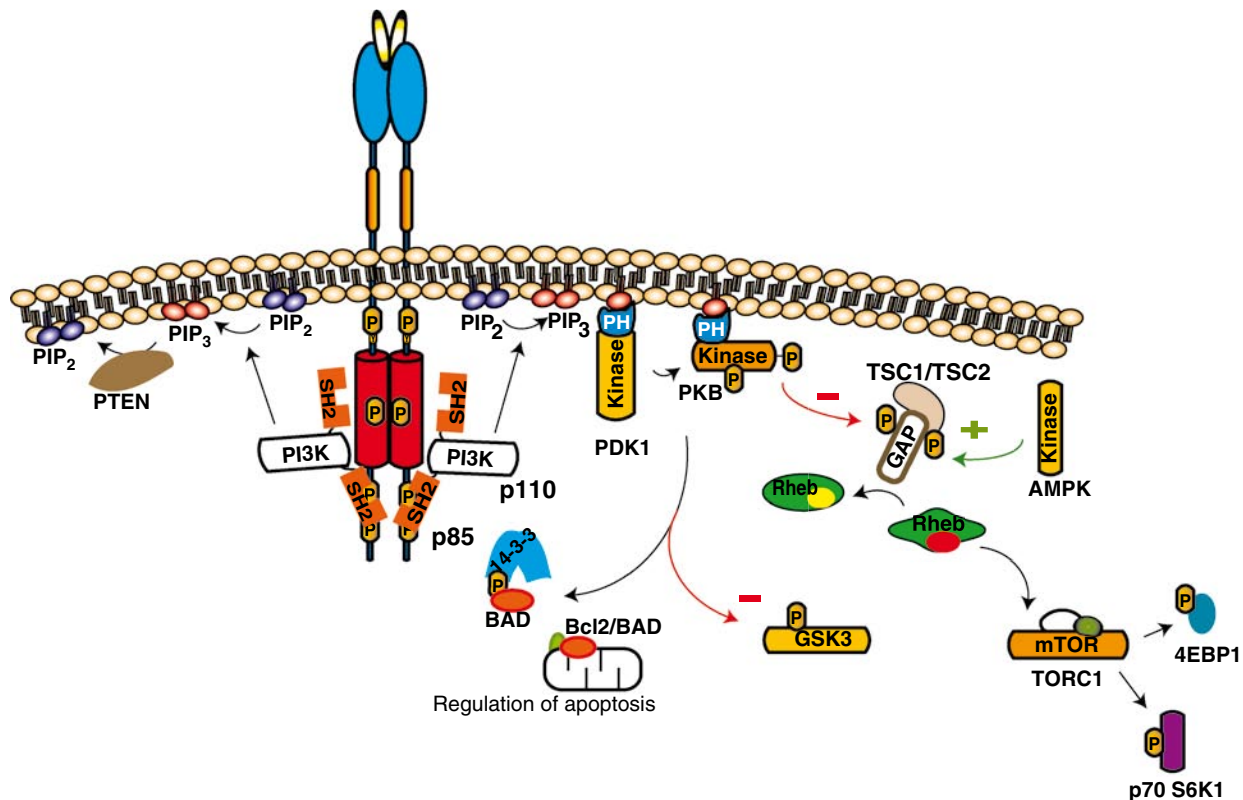


FIGURE 11-6 ACTIVATION OF PHOSPHATIDYLINOSITOL-3-KINASE (PI3K) BY RECEPTOR TYROSINE KINASES. Activation of receptor tyrosine kinases generates recognition motifs for the SH2 domains of the p85 regulatory subunit, which recruits the class IA PI3K to the membrane. Production of PIP₃ results in membrane association of various PH domain-containing proteins, such as PDK1 and Akt/PKB. PDK1 phosphorylates and activates Akt/PKB. Active Akt/PKB inactivates TSC1/TSC2 and therefore allows GTP-bound Rheb to regulate the TORC complexes. In addition, Akt/PKB phosphorylates BAD, generating a 14-3-3 recognition motif resulting in cytoplasmic retention of BAD. The phosphatase PTEN dephosphorylates PIP₃, leading to signal termination.

domains to interact, promoting a conformational change from an autoinhibited to an active state.

The distinction between inactive and active conformations is an important one for the design of tyrosine kinase inhibitors, since these can act by stabilizing the inactive conformation (e.g., Gleevec) or directly inhibiting the active state (e.g., Desatinib; 75,76). Many human tumors contain mutations in the genes encoding RTKs, which generally induce receptor activation by mimicking the dimeric (or oligomeric) state. As an example, pairs of cysteine residues in the extracellular immunoglobulin (Ig)-like domains of RTKs normally form intramolecular disulfide bonds. However, when one of these cysteines is mutated, the remaining residue forms an intermolecular disulfide bond with the corresponding cysteine of a neighboring chain, thus locking the receptor in a constitutively active dimeric configuration, as occurs with the Ret RTK in multiple endocrine neoplasia type 2A (77).

Recruitment of Cytoplasmic Targets to Receptor Tyrosine Kinases

Once activated, the RTK phosphorylates multiple tyrosine sites that lie outside the kinase domain, for example in the juxtamembrane region or C-terminal tail of the receptor, through transautophosphorylation. Upon phosphorylation, these motifs become docking sites for proteins with SH2 domains, which typically recognize the phosphorylated tyrosine and the following three to five amino acids with a dissociation constant in the range of 0.5 to 5 μ M (18,19). Different SH2 domains have distinct preferences for the residues flanking the pTyr, and the sequences surrounding a receptor's autophosphorylation sites can therefore have a marked influence on the specific SH2-containing cytoplasmic targets that it recruits (78). For example, the SH2 domain of Grb2 adaptor protein binds preferentially to sites in which a pTyr is followed by two residues to the C-terminal side by an Asn (the +2 position), whereas the two SH2 domains of the p85 adaptor subunit of PI3K bind selectively to pTyr sites with a +3 Met. SH2 domains are found in adaptor proteins such as Grb2, which often link through SH3 domains to specific regulatory targets (Figures 11-2A, 11-2B, and 11-5). Alternatively SH2 domains can be intrinsic components of effectors such as phospholipase C- γ , which hydrolyzes PI(4,5)P₂ to inositol triphosphate and diacylglycerol, in turn stimulating calcium release (IP3) and activation of the serine/threonine kinase, protein kinase C (20).

SH2-containing proteins and their targets have a number of biochemical properties, through which RTKs are connected to cellular responses. These include GEFs for GTPases of the Ras, Rho, Rap, and Rab families, which upon binding to GTP can regulate a wide range of pathways that communicate with the nucleus, the cytoskeleton, cell adhesion, and vesicle trafficking. SH2 proteins can also link RTKs to phosphoinositide metabolism (i.e., PI3K, PLC- γ), directly to transcriptional control through the STAT proteins, to cytoskeletal components, and to secondary tyrosine phosphorylation through Src family tyrosine kinases and SH2-containing tyrosine phosphatases.

In addition to recruiting activators of signaling, RTKs also bind proteins that attenuate specific pathways (such as Ras-GAP)

or down-regulate the receptor itself (such as the c-Cbl E3 protein-ubiquitin ligase). Variants of the Met RTK, the receptor for hepatocyte growth factor, have been identified in human lung cancer and found to have a deletion that removes the binding site for the Cbl SH2 domain. This leads to decreased receptor ubiquitination, abnormally prolonged residency of an activated receptor at the cell surface, and enhanced downstream signaling (79,80). These data indicate that suppressing RTK down-regulation can stimulate malignant transformation.

Signaling by the Insulin Receptor

The receptor for insulin is a tyrosine kinase, but differs from other growth factor receptors in that the mature receptor is a preformed heterotetramer (composed of two α chains and two β chains, linked through intermolecular disulfide bonds), even in the inactive state (9). Insulin stimulates intermolecular autophosphorylation of the receptor β subunit, possibly by reorienting the kinase domains into a mutually productive conformation to induce phosphorylation of the activation segment. A key autophosphorylation site in the insulin receptor β subunit is Tyr960, in the juxtamembrane region. Upon phosphorylation, this site binds to the PTB domain of the docking protein IRS-1, which consequently is phosphorylated at several tyrosines in YXN and YXXM motifs (81,82). These phosphorylated IRS-1 sequences are then recognized by the SH2 domains of Grb2 and PI3K, leading to activation of their downstream pathways (see following section). Signaling by the insulin receptor therefore involves a series of pTyr-dependent protein-protein interactions and the recruitment of a docking protein to activate intracellular signaling (83,84).

Cytoplasmic Tyrosine Kinases

Like RTKs, cytoplasmic tyrosine kinases are also important in normal signaling (Figure 11-1) and can be inappropriately activated as a consequence of oncogenic mutations. In the case of the T-cell antigen receptor (TCR), the α/β receptor chains, which bind antigen in the context of MHC, are linked to nonpolymorphic signaling subunits. Upon antigen engagement, these become phosphorylated by Src family kinases (Lck and Fyn) on motifs that contain two pTyr sites (so-called immunoreceptor activation motifs [ITAMs]). Once phosphorylated on both tyrosines, ITAMs bind the tandem SH2 domains of the ZAP-70 tyrosine kinase, which stimulates a series of signaling pathways leading to T cell activation (85-87). The importance of Src family kinases and ZAP-70 in T-cell signaling, and of the pTyr-dependent SH2 domain interactions in this process, has been demonstrated through genetic analysis in mice and the discovery of mutations that cause human immunodeficiencies (88-90).

Although Src family kinases and ZAP-70 are not known to be consistently mutated in human cancers, the related cytoplasmic kinase Abl is a characteristically activated in chronic myelogenous leukemia in the form of the chimeric Bcr-Abl oncoprotein encoded by the 9;22 Philadelphia chromosome (91,92). The N-terminal region of Bcr forms a tetramer, which drives intermolecular autophosphorylation of the Abl kinase domain, resulting in enhanced

kinase activity (93). This overcomes the autoinhibitory effects of the SH2 and SH3 interaction domains, which suppress catalytic activity through intramolecular interactions with the kinase domain. Bcr also provides a novel site (Tyr177) for autophosphorylation by the linked Abl kinase, which forms an ideal motif for binding the Grb2 SH2 domain (pYVNV), and the activation of Grb2 targets, thereby contributing to leukemic potential (94,95). Thus, Bcr-Abl corrupts cellular signaling through unscheduled tyrosine phosphorylation and aberrant protein–protein interactions.

In a related fashion to Lck and ZAP-70, JAK tyrosine kinases associate through their noncatalytic N-terminal region with the signaling subunits of cytokine receptors, which themselves lack intrinsic catalytic activity. Binding of a cytokine (e.g., interleukin-3, erythropoietin) to its receptor induces JAK autophosphorylation, likely by clustering of JAK kinase chains to allow transautophosphorylation (96–98). The activated JAK kinase then phosphorylates sites in the tail of the cytokine receptor that selectively recruit the SH2 domain of STAT transcription factors. Once associated with the receptor–JAK complex, STAT proteins are phosphorylated at a specific tyrosine residue, which stabilizes a dimeric form of STAT through mutual binding of the SH2 domain of one STAT molecule to the pTyr site (99,100). Phosphorylated STAT dimers move to the nucleus, where they engage the promoters of their transcriptional targets through a DNA-binding region adjacent to the SH2 domain, thus inducing specific gene expression.

Among the gene products up-regulated by STAT transcription factors are SH2-containing polypeptides termed “SOCS proteins” (101). Once synthesized, these can bind the activated receptor and terminate signaling by direct inhibition of JAK kinase activity and promoting ubiquitination. This is a further example of a negative feedback, which ensures that signaling is transient.

The JAK2 kinase interacts with numerous members of the cytokine family of receptors, including those for growth hormone (GH), prolactin, erythropoietin (EPO), γ -interferon, and leptin. JAK2 sustains a specific mutation in a range of myeloproliferative disorders, such as polycythemia vera, and some acute leukemias. The resulting substitution (V617F) is in a noncatalytic kinase-like domain and likely disrupts an autoinhibitory interaction (102,103).

Intracellular Signaling Pathways

The JAK-STAT pathway discussed in the preceding section represents the shortest route by which a cell surface receptor can communicate with its ultimate intracellular target, in this case by directly controlling a transcription factor. Here, we introduce two more extended pathways of great importance for the control of cell growth, division, survival, and differentiation by growth factor receptors. Members of these pathways are common targets of gain- or loss-of-function mutations in cancer.

The Ras-MAP Kinase Pathway

MAPKs play a central role in signal transduction in eukaryotic cells. They are present in simple organisms such as yeast, where they

respond to extracellular signals that control the cell cycle, mediate pathways activated by environmental stress, and regulate cell invasion. MAPKs are activated by the phosphorylation of threonine and tyrosine residues, located in a TXY motif in the activation segment of their kinase domain. This motif is phosphorylated by an upstream dual-specificity protein kinase (MAP kinase kinase or MAPKK), which is activated by a MAP kinase kinase kinase or MAPKKK (Figure 11-5; 104,105). In the yeast *Saccharomyces cerevisiae*, the mating pheromone signals through a GPCR to activate a MAPKKK (Ste11), a MAPKK (Ste7), and two MAPKs (Fus3 and Kss1). The fidelity of this pathway is maintained, in part, by the scaffolding protein Ste5, which binds all kinases in the pathway (106). The activated yeast MAP kinases control cellular function through the phosphorylation of transcription factors, such as Ste12.

Conventional tyrosine kinases are absent from yeast, and first appear in the immediate predecessors of multicellular animals. This has led to the hypothesis that RTK signaling provided an important evolutionary advance in communication between cells, which allowed for metazoan species to emerge. As part of this process, RTKs have developed mechanisms to activate MAPK pathways. In the prototypic example (Figure 11-5), activated RTKs become autophosphorylated at YXN motifs; these bind the central SH2 domain of the Grb2 adaptor, which has two flanking SH3 domains. The SH3 domains of Grb2 engage proline-rich sequences in the C-terminal tail of Sos proteins, which act as GEFs for Ras GTPases (107,108). This recruitment of the Grb2–Sos complex to autophosphorylated RTKs results in activation of Ras at the plasma membrane, by the exchange of GDP for GTP. Of interest, Ras-GTP can bind Sos and enhance its activity, indicating a positive feedback loop that can promote Ras GTP-loading (109).

The Ras family has three members, H-, N-, and K-Ras, all of which are post-translationally modified by a form of fatty-acid lipidation, isoprenylation, on Cys-186 and are consequently attached to membranes. H- and N-Ras are also palmitylated, which further promotes their interaction with the plasma membrane, while K-Ras has a run of basic amino acids that can interact with membrane phospholipids (110). The conformational change experienced by Ras proteins on binding to GTP affects two regions (termed “switch 1” and “switch 2”), encompassing residues 30 to 38 and 60 to 76 (111). In the active conformation, the switch regions of Ras interact with a series of target proteins, most importantly the Raf serine/threonine–protein kinases (A-, B- or c-Raf; 112,113). Raf proteins are MAPKKKs and thus phosphorylate and activate a MAPKK (MEK), which regulates the ERK MAPK (of which there are two isoforms, ERK1 and ERK2). There is significant amplification through the pathway, such that activation of only a small fraction of Ras molecules can elicit full activation of ERK1/2 (114).

Raf protein kinases have an N-terminal regulatory region, followed by the catalytic domain and a short C-terminal tail. As befits their location at the apex of the ERK MAPK cascade, the activity of Raf kinases is subject to multiple controls (115). Raf is held in an inhibited state by phosphorylation of two Ser residues in the N- and C-terminal regions of the kinase, with each

phosphorylated site recognized by a 14-3-3 protein (Figure 11-5; 116). The 14-3-3 proteins bind selectively to specific phosphoserine/threonine sites and form dimers such that each dimer has two phosphopeptide-binding sites. In the case of Raf proteins, a 14-3-3 dimer can bridge the N- and C-terminal Raf phosphorylation sites to promote an inactive kinase conformation. Activation of Raf protein kinases involves the binding of Ras-GTP to a site in the N-terminal regulatory region, dephosphorylation of the N-terminal 14-3-3-binding site by the phosphatase PP2A, and phosphorylation within the activation segment of the kinase domain (117–119). This process also requires the cytoplasmic scaffolding protein KSR (Figure 11-5), which interacts with both regulators of the Raf kinase (14-3-3 proteins, PP2A) and its substrate MEK (120,121). The c-Raf isoform also requires additional activating phosphorylating events on Ser and Tyr residues in the N-terminal region, which are replaced by acidic amino acids in the B-Raf isoform (122). The fact that B-Raf is less tightly regulated than c-Raf may be relevant to the finding that B-Raf is commonly activated in a number of human cancers (see subsequent section), whereas c-Raf is only infrequently mutated.

The distal target of this pathway, the ERK MAPK, has multiple substrates that regulate cell growth and entry into the cell cycle, including transcription factors such as c-Fos, Myc, and Elk1 and downstream protein kinases, including p90 Rsk (ribosomal protein S6 kinase) and Mnk (MAPK-interacting protein kinase; 104,123). An important feature of signaling by ERK MAPKs is the presence of specific docking sites in their substrates and regulators (for example the “D” and DEF domains, the latter with the sequence FXFP), which interact with the ERK kinase at regions distant from the active site. This increases the specificity with which substrates are recognized by providing at least two contact sites between the kinase and its targets: a noncatalytic docking interaction followed by recognition of the phosphorylation site at the active site (124,125).

Consistent with the notion that the Ras-MAPK pathway is important in mitogenic signaling from RTKs, members of the Ras and Raf families frequently undergo oncogenic mutations in cancer (126,127). In the case of Ras GTPases, substitutions at residues 12, 13, 59, or 61 (most notably Gly12 and Gln61) result in Ras becoming trapped in the GTP-bound state, owing to a loss of intrinsic GTPase activity and decreased sensitivity to GAP-stimulated GTP hydrolysis. In this constitutively active state, Ras can interact with target proteins such as Raf in the absence of an upstream signal from the RTK–Grb2–Sos complex. Such activating Ras mutations have been detected in about 30% of human cancers (128). An alternative mechanism through which Ras GTPase might be activated is through loss of inhibitory GAP activity; indeed the neurofibromin 1 (NF1) tumor suppressor is a Ras–GAP, and its inactivation leads to elevated levels of Ras-GTP (129).

Activating mutations in B-Raf were recently identified in a number of cancers, most prominently in more than 60% of malignant melanomas, as well as in colon, thyroid, and lung cancers (127). The resulting substitutions are located in the kinase domain and map to the activation loop in the large lobe or the P loop in the small lobe of the kinase (the latter involved in ATP

binding). Structural analysis has revealed that these two elements are associated when B-Raf is in the inactive state, which promotes a closed conformation of the small and large lobes of the kinase domain, which restricts catalytic activity (130). The cancer-related mutations disrupt this inhibitory interaction and thereby stimulate kinase activity and MEK phosphorylation, resulting in aberrantly high ERK MAPK activity. Strikingly, some transforming B-Raf mutations promote the open, activated conformation of the kinase, but also suppress enzymatic activity. These B-Raf variants can apparently signal by binding and stimulating the c-Raf isoform, indicating that different Raf kinases can form active heterodimers (131).

Although the Ras-MAPK pathway can be portrayed in a linear fashion, it has multiple potential branch points. In particular, in addition to Raf, Ras-GTP can interact with a several proteins that share a Ras-binding domain (RBD). These include proteins such as the p110 catalytic subunit of PI3K (see subsequent section), RalGDS (a GEF for the Ral GTPase), PLC ϵ , and TIAM1 (a GEF for the Rac and Rho GTPases). Thus Ras can potentially stimulate multiple pathways, which may all participate in generating the full cellular response (132). In support of this view, Ras-GTP binding partners such as RalGDS, PLC ϵ , and TIAM1 can contribute to Ras-induced carcinogenesis in mice (133–135).

The PI3K Pathway

As noted previously, a prominent pathway activated by many RTKs involves the phosphorylation of the inositol head group of PI(4,5)P₂ on the D3 position, to generate PI(3,4,5)P₃. The PI3Ks that function downstream of growth factor receptors (Figure 11-6) possess an adaptor subunit (p85) and a catalytic subunit (p110), and constitute the IA class of the PI3K family. The p85 subunit has two SH2 domains, with a preference for phosphorylated YXXM sites (136), flanking a sequence that binds the p110 catalytic subunit. Engagement of the p85 SH2 domains is sufficient to stimulate p110 PI3K activity, resulting in the production of PI(3,4,5)P₃ from PI(4,5)P₂ at the plasma membrane (137,138). PI(3,4,5)P₃ can be converted back to PI(4,5)P₂ by a specific lipid phosphatase, PTEN, which antagonizes PI3K signaling (Figure 11-6; 139). PIP₃ exerts its effects by binding to the PH domains of specific proteins, which are thereby localized to PIP₃-rich regions of the plasma membrane (140). Notable among these PH domain-containing proteins are the serine/threonine-protein kinases PKB (Figure 11-2B; also called Akt) and PDK1. PKB is recruited to the membrane by association of its PH domain with PIP₃ (141,142) and is activated through phosphorylation at Ser473 in its C-terminus (likely by the mammalian target of rapamycin [mTOR]; see subsequent section) and in the activation segment of the kinase domain at Thr308 by PDK1 (Figure 11-6; 143–145). PKB, once activated, has multiple substrates that control cell growth, passage through the cell cycle, survival, and metabolism.

A critical pathway to cell growth involves the ability of PKB to phosphorylate and inhibit the TSC2 protein (tuberin), which in complex with TSC1 (hemartin) acts as a GAP for the Rheb GTPase (146–148). GTP-bound Rheb activates the mTOR

protein kinase, a central regulator of protein synthesis, which is regulated by intracellular nutrient availability, ATP levels, and extracellular signals through the PI3K pathway. The cellular concentration of ATP is monitored by a protein kinase that is activated by AMP, a metabolite of ATP. AMP-activated kinase phosphorylates and stimulates the TSC2/TSC1 Rheb GAP, thereby blocking the ability of Rheb to activate the mTOR complex (149). Thus AMPK and PKB have opposing effects on mTOR. PKB leads to its activation in response to an extracellular growth-promoting signal, whereas AMPK suppresses its activity when the cell is depleted of the energy required for new protein synthesis.

mTOR is a large protein with an extended N-terminal non-catalytic region that binds partners such as Raptor and Rictor. These latter proteins act as adaptors to target mTOR to specific substrates, which they bind through a specific peptide motif reminiscent of the docking domains for ERK MAPK substrates. Binding of Raptor or Rictor to mTOR is mutually exclusive and yields the mTORC1 and mTORC2 complexes, respectively, which have distinct biologic activities (150). Two of the well-characterized targets of mTORC1 are 4EBP1 and S6K (Figure 11-6; 151,152). 4EBP1 is an inhibitor of protein synthesis through its ability to bind and block the translation initiation factor eIF4E; phosphorylation of 4EBP1 by PKB causes its release from eIF4E, which is thereby liberated to stimulate protein synthesis. mTORC1 also phosphorylates and activates S6K1 in a fashion that promotes translational initiation.

Another important target of activated PKB is the regulatory machinery that controls cell survival (153). This includes phosphorylation of the pro-apoptotic protein BAD, which consequently binds 14-3-3 proteins and is blocked from its inhibitory association with the anti-apoptotic Bcl2 polypeptide (Figure 11-6; 154–156). PKB also phosphorylates FOXO transcription factors at sites that bind 14-3-3, resulting in FOXO protein being in the cytoplasm. This inhibits the ability of FOXO proteins to induce the expression of pro-apoptotic and stress-response genes.

In addition, PKB phosphorylates and inactivates the GSK-3 protein kinase, which is itself an inhibitor of cell cycle regulators (157,158). PKB can also phosphorylate metabolic enzymes such as 6-phosphofructo-2-kinase and ATP citrate lyase and thereby control glycolysis and fatty-acid synthesis. PKB and mTOR are hub proteins that interact with multiple targets and accessory factors and consequently have complex and pleiotropic effects on cellular function. In the case of the mTORC1 complex, the small-molecule rapamycin nucleates an additional, nonphysiologic interaction with the FKBP12 protein, which inhibits mTOR activity.

Mutations that affect components of the PI3K pathway are involved in many human cancers (153,159). These include activating mutations in PIK3CA (p110 α ; 160) and loss-of-function mutations in the phosphatase PTEN, both of which drive the aberrant formation of PIP₃. Inactivating mutations in TSC1 and TSC2 also result in familial cancer syndromes as do loss-of-function mutations in LKB1, a protein kinase that stimulates AMPK, and therefore normally antagonizes PI3K-mTOR signaling (161). Taken together, these data argue that the PI3K pathway is important for the normal regulation of cell growth and proliferation in response to growth factor stimulation and is inappropriately activated in cancer cells.

Summary

Growth factor receptors use a series of switchlike molecular devices, including phosphorylation, regulated protein–protein interactions, GTP-binding to Ras-like proteins, and targeted proteolysis to control a range of cellular responses that promote cell proliferation and survival. Several other types of receptors and signaling pathways modify the behavior of human cells, including receptors for proteins in the Wnt, Hedgehog, and Delta families. Although many details are different, the basic molecular devices described above for RTKs are also used by these other cell surface receptors.

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Cell Growth

What is “Cell Growth?”

Cell growth is the process by which cells accumulate mass and increase in physical size. On average, animal cells are ≈ 10 to $20\ \mu\text{m}$ in diameter with a wide range of sizes, spanning from tiny red blood cells ($\approx 5\ \mu\text{m}$ in diameter) to motor neurons, which can grow 100's of micrometers in length (1). Water accounts for $\approx 70\%$ of the weight of a cell and macromolecules, such as nucleic acids, proteins, polysaccharides, and lipids constitute most of the rest ($\approx 25\%$; trace amounts of ions and small molecules make up the difference). The largest contribution to animal cellular dry mass is from proteins, which makes up about 18% of the total cell weight on average. There are many physical, chemical, and biologic factors that affect final cell size. However, it is the biologic factors, in particular intracellular signaling networks that control macromolecule synthesis, that are immediately relevant to cancer. As discussed in the following sections, deregulation of the cellular circuitry controlling biomass accumulation is associated with a wide spectrum of human cancers.

In some cells, size is proportional to DNA content. For instance, continued DNA replication in the absence of cell division (called endoreplication) results in increased cell size. Megakaryoblasts, which mature into granular megakaryocytes, the platelet-producing cells of bone marrow, typically grow this way. These cells cease division then undergo multiple rounds of DNA synthesis, increasing from ≈ 20 to $\approx 100\ \mu\text{m}$ in diameter as a result of the increased DNA content. It is unclear whether increased DNA content simply leads to an increase in total cellular material or whether cells actively grow to cope with the larger genome size. This growth strategy is found throughout nature in animals, plants, and single-celled organisms. By a different strategy, adipocytes can grow to ≈ 85 to $120\ \mu\text{m}$ by accumulating intracellular lipids. In contrast to endoreplication or lipid accumulation, some terminally differentiated cells, such as neurons and cardiac muscle cells, cease dividing and grow without increasing their DNA content. These cells proportionately increase their macromolecule content to a point necessary to perform their specialized functions. This involves coordination between extracellular cues from nutrients and growth factors and intracellular signaling networks responsible for controlling cellular energy availability and macromolecular synthesis.

Perhaps the most tightly regulated cell growth occurs in dividing cells, where cell growth and cell division are clearly separable processes. Dividing cells generally must increase in size with each passage through the cell division cycle to ensure that a consistent average cell size is maintained. (There are examples in the animal kingdom where cell division in the absence of growth serves an important evolutionary function, such as during the syncytial division stage of the early developing *Drosophila* embryo.) For a typical dividing mammalian cell, growth occurs in the G_1 phase of the cell cycle and is tightly coordinated with S-phase (DNA synthesis) and M phase (mitosis). The combined influence of growth factors, hormones, and nutrient availability provides the external cues for cells to grow. It is hypothesized that once a threshold cell size is attained, cells irreversibly commit to at least one round of division, thus achieving adequate size is a prerequisite for DNA synthesis and mitosis. Deprivation of nutrients and other growth signals, as might be the case in the nutrient-, and oxygen-, starved regions of an advancing tumor, may encourage normal cells to exit the cell cycle into a resting or G_0 state. Mutations resulting in deregulation of a cell's ability to sense nutrients or growth factors may thus provide tumor cells with a selective growth advantage. Efforts to identify intracellular signaling networks that control growth are therefore a mainstay of many cancer-focused research programs.

Biochemical Pathways that Control Growth

The Mammalian Target of Rapamycin-1 Pathway

Essential to connecting cell growth control with cancer pathogenesis was the identification of intracellular signaling molecules that coordinate signals from nutrient availability, growth factors, and hormones with autonomous cell growth. The most prominent intracellular regulator of growth is a large protein kinase called TOR (target of rapamycin; 2,3). TOR was discovered as the molecular target of rapamycin, an antifungal and immunosuppressive drug originally isolated in the 1970s from soil bacteria living on Easter Island (Rapa Nui). Rapamycin is also recognized for its

ability to prevent restenosis after angioplasty and potential as an anticancer therapeutic. Rapamycin binds an intracellular receptor protein called FKBP12 and the rapamycin–FKBP12 complex binds potently and specifically to TOR. Extensive studies largely done in yeast, *Drosophila*, and mammalian cultured cells has unveiled a complex TOR–centric signaling network responsible for converting extracellular growth signals into a growth response.

The ability of mammalian TOR (mTOR) to control growth relies on its association with the regulatory protein raptor (Figure 12-1). Raptor appears to function as a regulator of mTOR catalytic activity and as a scaffold for recruiting mTOR substrates. The complex also contains a protein of unknown function called mLST8 (also known as Gβ1), and collectively this heterotrimeric complex is referred to as mTORC1 (mTOR complex 1). mTORC1 is directly bound by rapamycin–FKBP12, and although the drug destabilizes the association between mTOR and raptor, it does not dissociate any components of the complex leaving the mechanism of rapamycin function to be fully explained. Largely as a result of genetic studies in yeast and experiments with rapamycin in cultured cells, it is widely accepted that mTORC1 controls an array of cellular processes, including mRNA translation, ribosome biogenesis, metabolism, transcription of growth regulatory genes, and autophagy—all of which have roles in cancer pathogenesis. However, only some of the intermediate regulatory molecules linking mTOR with these processes are known.

Although its precise mechanism of inhibiting mTORC1 remains elusive, rapamycin clearly prevents mTOR from phosphorylating the two most extensively characterized mTORC1 substrates, the S6 kinase 1 (S6K1) and eIF-4E-binding protein 1 (4E-BP1), both of which regulate protein synthesis. Following coregulatory phosphorylation by mTOR and another kinase called phosphatidylinositol 3–dependent kinase 1 (PDK1), S6K1 is believed to positively affect mRNA synthesis by phosphorylating translational regulators such as ribosomal protein S6 and eIF-4b. Evidence suggests S6K1 activation may involve its release from the eIF3 preinitiation complex following mTORC1 recruitment (4). Better understood is the function of 4E-BP1, which in the unphosphorylated state binds and inhibits the translational regulator eIF4E. When phosphorylated by mTOR, 4E-BP1 is relieved of its inhibitory duty, promoting eIF4E-dependent translation of capped nuclear transcribed mRNA. Although the preponderance of evidence indicates that S6K1 and 4E-BP1 are directly phosphorylated by mTOR, it cannot be ruled out that another kinase or an mTOR-inhibited phosphatase may be involved. Contributing to this debate is the fact that the rapamycin-sensitive phosphorylation sites on S6K1 and 4E-BP1 lack any sequence similarity, a trait that has challenged efforts to identify other mTORC1 substrates that could mediate ribosome biogenesis, transcription, or autophagy.

The connection between cell growth control by mTORC1 and cancer has solidified with the identification of upstream mTORC1 regulators. Building mass requires adequate metabolic building blocks, sufficient energy, and favorable environmental conditions. Therefore, it is not surprising that mTORC1 activity is controlled by numerous factors including amino acid and glucose availability, growth factors, ATP amount, mitochondrial

activity, and oxygen levels, all of which are needed for a cell to grow. Understanding how signals derived from these diverse factors influence mTORC1 activity is an intense area of investigation. However, the discovery in *Drosophila*, mice, and human cultured cells that the two components of the tuberous sclerosis complex (TSC), TSC1 (hamartin) and TSC2 (tuberin), function together to negatively regulate mTORC1 activity may have provided a key step to unraveling the mystery. As a bipartite complex with GTPase activity, TSC1/2 suppresses the activity of the Rheb GTP-binding protein, which is reported to bind and activate mTORC1 (Figure 12-1). Mutation in the TSC1 or TSC2 gene results in aberrant up-regulation of mTORC1 activity as measured by S6K1 phosphorylation and can cause a devastating tumor-prone human disease called tuberous sclerosis (described in subsequent sections).

Models indicate the TSC1/2 heterodimer integrates many of the signals responsible for modulating mTORC1 activity (2;5–7). Importantly, TSC1/2-dependent regulation of mTORC1 can be differentially modified, both positively and negatively, depending on growth conditions. For instance, a drop in adenosine triphosphate (ATP) levels (i.e., energy deprivation) can trigger direct phosphorylation of TSC2 by AMPK, a sensor of the cellular ATP: AMP ratio, promoting TSC1/2 to inactivate mTORC1 and halt growth. Oxygen deprivation (hypoxia) similarly inactivates mTORC1 through TSC1/2 but by a different mechanism involving HIF-dependent expression of REDD1 and REDD2. Nutrient deprivation, particularly of amino acids, also deactivates mTORC1 in cells, although the nature of the signal and whether it requires TSC1/2 are not known. Conditions that negatively impact growth, such as decreased energy, low oxygen, and insufficient nutrients, are associated with the harsh environment of a developing or poorly vascularized tumor. The ability of cancer cells to overcome these adverse conditions would promote tumor growth, putting the desensitization of mTORC1 signaling in the spotlight as a potential mechanism cancer cells could use to enhance their viability.

Conditions positively impacting growth, such as signals emanating from growth factor receptors, can also signal through TSC1/2. Inhibitory phosphorylation of TSC2 resulting from activation of the MAPK/ERK pathway has been reported. However, the best-described example of growth factor regulation of TSC1/2 is through the PI3K–Akt/PKB pathway. Direct phosphorylation of TSC2 by the Akt/PKB protein kinase appears to inhibit TSC1/2 in a variety of systems and thereby promote activation of mTORC1. Akt/PKB is a critical effector of the phosphatidylinositol-3–kinase (PI3K) growth-factor–signaling pathway. Following stimulation by growth factors, such as insulin, IGF-1, or PDGF, receptor tyrosine kinases activate PI3K, which subsequently phosphorylates membrane associated phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) to generate phosphatidylinositol-3,4,5-triphosphate PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ serves as a docking site for the membrane-recruitment and activation of Akt/PKB. The phosphatase PTEN, the second most frequently mutated tumor suppressor (after p53), balances PI3K activity by dephosphorylating PtdIns(3,4,5)P₃ and thus negatively regulates Akt/PKB activity.

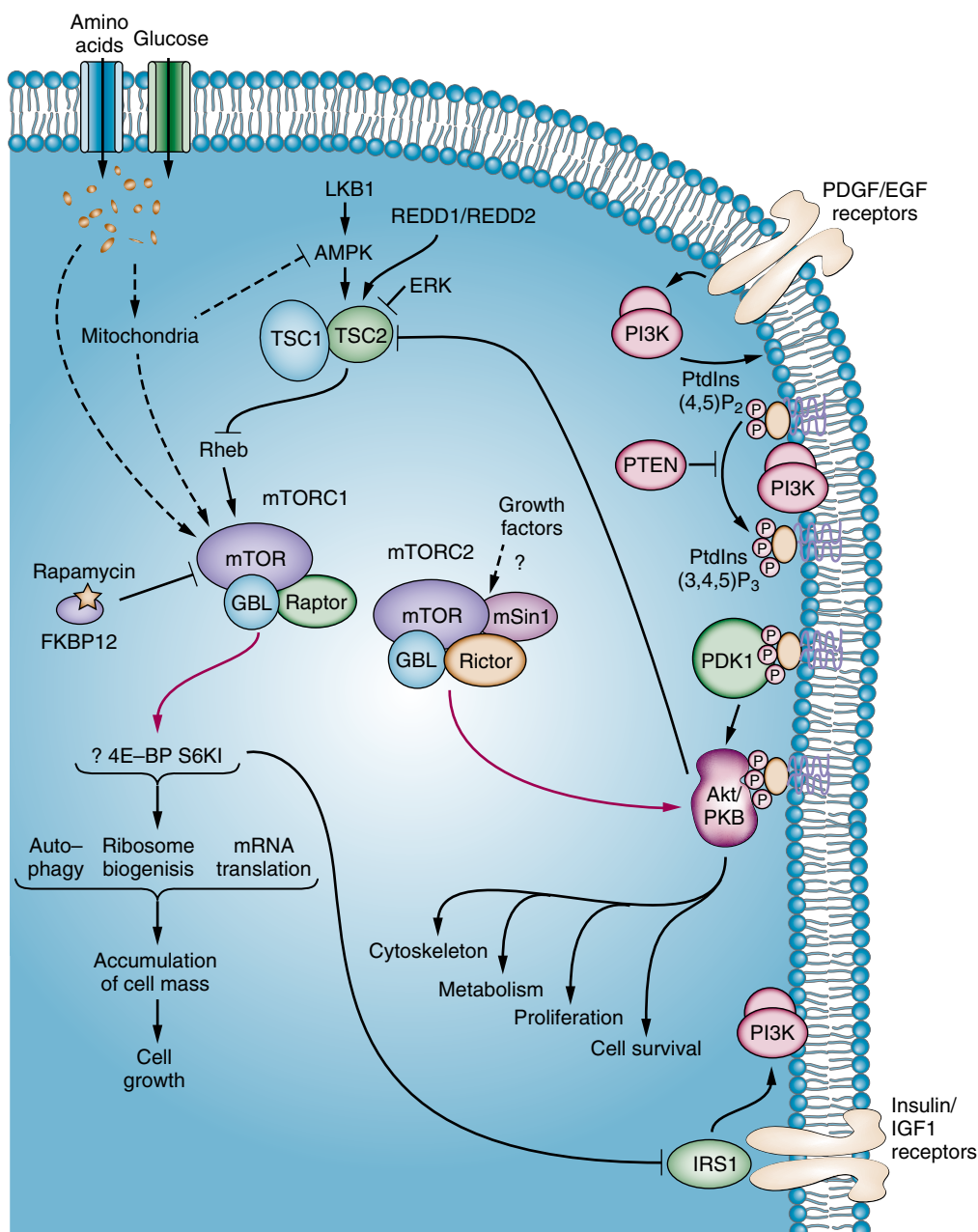


FIGURE 12-1 Current model of the mammalian target of rapamycin (mTOR) network. mTOR is the catalytic subunit of two distinct signaling complexes called mTORC1 (**left**) and mTORC2 (**right**). In addition to mTOR, mTORC1 contains the raptor and mLST8 proteins. mTORC2 also contains mLST8, but instead of raptor, this complex contains the rictor and mSin1 proteins. mTORC2 signaling controls cell growth in part by phosphorylating the S6 kinase and 4E-BP1 proteins (*left red arrow*). mTORC2 controls cell survival, proliferation, metabolism, and aspects of the cytoskeleton by phosphorylating the Akt/PKB kinase (*right red arrow*). mTORC2 regulation of Akt/PKB occurs with growth factor signaling through the phosphatidylinositol-3-kinase (PI3K) pathway. Upstream regulators of mTORC1, such as the TSC1/TSC2 complex, have recently been discovered. However, it is speculated that other inputs, particularly from nutrients, regulate mTORC1 activity by an unknown mechanism (*dotted arrows*). It is not known how mTORC2 activity is regulated but growth factors are suspected to play an important role. (From Sarbassov dos D, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 2005;17:596–603, with permission.)

PI3K/Akt Signaling, mTORC1, and Cancer

Mutations causing loss of PTEN function or oncogenic activation of PI3K or Akt/PKB are associated with many aggressive human cancers (Table 12-1;8,9). The finding that Akt/PKB can phosphorylate and inhibit TSC1/2, and thus activate mTORC1, has led to speculation that cancers with elevated PI3K/Akt signaling may thrive due in part to an mTORC1-driven growth advantage. The

contribution and relevance of mTORC1 downstream functions in cancers with aberrant PI3K-Akt/PKB signaling is not known, but interestingly overexpression of eIF4E (the negatively regulated target of the mTORC1 substrate 4E-BP1) can transform cells. Unlike tumor cells characterized by loss of PTEN function, tumor cells having lost TSC1/2 function, which biochemically is several steps closer to mTORC1 regulation, are generally less belligerent.

Table 12-1 mTOR Signaling in Disease

Disease	Linked Genetic Mutation and Clinical Pathology	Predicted Functional Link to mTOR Signaling
Tumor-prone syndromes		
TSC (tuberous sclerosis complex)	<i>TSC1</i> or <i>TSC2</i> ; hamartomas in multiple organs	<i>TSC1/2</i> negatively regulates Rheb
LAM (lymphangioleiomyomatosis)	<i>TSC2</i> ; abnormal proliferation of smooth muscle-like cells in the lung	<i>TSC1/2</i> negatively regulates Rheb
Cowden disease	<i>PTEN</i> ; hamartomatous tumor syndrome	may promote AKT-dependent inhibition of <i>TSC2</i> and mTOR phosphorylation
Proteus syndrome	<i>PTEN</i> ; hamartomatous tumor syndrome	may promote AKT-dependent inhibition of <i>TSC2</i> and mTOR phosphorylation
Lhermitte-Duclos disease	<i>PTEN</i> ; hamartomatous tumor syndrome	may promote AKT-dependent inhibition of <i>TSC2</i> and mTOR phosphorylation
PJS (Peutz-Jeghers syndrome)	<i>STK11/LKB1</i> ; gastrointestinal hamartoma tumor syndrome	<i>STK11</i> activates AMPK, a positive regulator <i>TSC2</i>
HCM (familial hypertrophic cardiomyopathy)	<i>AMPK</i> ; myocardial hypertrophy	<i>AMPK</i> promotes <i>TSC2</i> function
Cancer		
Prostate	<i>PTEN</i>	<i>PTEN</i> loss promotes AKT activation
Breast	<i>PTEN</i> ; <i>PI3K</i> , <i>AKT</i> , or <i>Her2/neu</i> amplification/hyperactivation	<i>PTEN</i> loss or gene amplifications promote AKT activation
Lung	<i>PTEN</i> ; <i>HER</i> amplification	<i>PTEN</i> loss or gene amplifications promote AKT activation
Bladder	<i>PTEN</i>	promotes AKT activation
Melanoma	<i>PTEN</i>	promotes AKT activation
Renal cell carcinoma	<i>PTEN</i>	promotes AKT activation
Ovarian	<i>PTEN</i> ; <i>PI3K</i> , <i>AKT</i> , or <i>Her2/neu</i> amplification/hyperactivation	<i>PTEN</i> loss or gene amplifications promote AKT activation
Endometrial	<i>PTEN</i>	promotes AKT activation
Thyroid	<i>PTEN</i> ; <i>PI3K</i> , <i>AKT</i> , or <i>Her2/neu</i> amplification/hyperactivation	<i>PTEN</i> loss or gene amplifications promote AKT activation
Brain (glioblastoma)	<i>PTEN</i>	promotes AKT activation
CML (chronic myeloid leukemia)	<i>BCR/ABL</i> translocation	promotes AKT activation

mTOR, mammalian target of rapamycin.

Source: Adapted from Guertin DA, Sabatini DM. An expanding role for mTOR in cancer. *Trends Mol Med* 2005;11:353–361.

This appears to be due to an inhibitory feedback loop through S6K1 that can suppress the PI3K-Akt/PKB pathway (10–12). When activated by mTORC1, S6K1 can directly phosphorylate and inactivate IRS1 and IRS2, two mediators of PI3K activation. This negatively affects the activity of PI3K and Akt/PKB in some cells and therefore is thought to squelch additional oncogenic cues necessary for transformation of *TSC1/2* defective tumors into an aggressive cancer.

mTORC1 and Autophagy

Macroautophagy, or simply autophagy, is a lysosome-dependent catabolic process induced on nutrient limitation or stress in which cells break down proteins and organelles into basic components that are recycled as sources of cellular energy and metabolites (13). Autophagy is predicted to have an important role in cell survival

during times of energy crisis by providing important biomaterials required for basic cell sustenance. Autophagy is also thought to salvage and recycle cellular junk such as excess or damaged organelles. The finding that rapamycin addition to mammalian cells or genetic inactivation of TOR in *Drosophila* induces autophagy indicates that mTORC1 plays an important negative role in controlling autophagy. The molecular intermediates linking mTORC1 to autophagy are unknown.

The strong connections between mTORC1 and pathways implicated in cancer suggest that autophagy has a role in suppressing carcinogenesis. Emerging evidence supports this hypothesis. For example, mice deficient for a pro-autophagy gene, called *beclin-1*, have an increased frequency of spontaneous tumor formation suggesting *beclin-1* is a tumor suppressor gene. How could inactivation of autophagy promote tumorigenesis? It is difficult to speculate at this time because little is known about Beclin-1, but perhaps some

transformed cells rely on autophagy to remove damaged organelles or for complete self-disposal. In this model, mutations that prevent autophagy would promote cell survival. Interestingly, the Bcl-2 prosurvival oncoprotein, which has long been thought to inhibit apoptosis, binds and inhibits Beclin-1-dependent autophagy (14). This suggests that the oncogenic activity of Bcl-2 might be linked to inhibiting nonapoptotic autophagic cell death. Contrary to having tumor suppressor capacity, autophagy could also promote tumorigenesis in some situations. When cancer cells experience nutrient limitation, autophagy may provide the rations necessary to sustain the most essential bioenergetic cellular process. This may allow cancer cells to survive until angiogenesis is initiated or other deleterious mutations occur. Understanding how autophagy is controlled by mTOR and the role of autophagy in different cancer cells will be important to designing mTOR-focused treatment strategies.

mTORC1 and *p53*

Mutation of the *p53* tumor suppressor is one of the most common genetic abnormalities associated with human cancer. DNA or spindle damage, telomere shortening, various stresses, or oncogenic mutations will usually initiate a *p53*-dependent checkpoint that arrests cells in G₁ or trigger apoptosis (depending on the cell type and signals present). This has led to speculation that such a checkpoint mechanism involving *p53* might communicate with the mTORC1 pathway to discourage growth when conditions are unfavorable. Early reports from cultured cells suggest AMPK and *p53* may communicate in some type of checkpoint mechanism to restrict growth in low-glucose conditions or when DNA is damaged, although it is unclear from this early work if and how directly mTORC1 regulation might be connected (15). Mounting evidence additionally indicates a communication link exists between *p53* and the regulation of mitochondrial respiration, particularly in mediating a cellular switch to preferentially deriving energy from glycolysis rather than aerobic respiration—a hallmark characteristic of cancer cells (16). It is well known that *p53* and the mitochondria, and to some extent mTOR, have roles in apoptosis. Because signals from the mitochondria are also thought to control mTORC1's ability to mediate growth, it seems likely that a complicated signaling circuitry involving both the *p53*- and mTOR-dependent pathway exists.

Does mTOR Regulate Organ and Organism Growth?

The mTORC2 Pathway

The role of TOR in building cell mass is well documented and conserved in all eukaryotic organisms examined. But extending a role for mTOR in controlling organ and organism growth is more complicated because most tissues grow by a mechanism involving the collective coordination of cell growth, cell division, and cell death pathways. However, a finding that mTOR exists in a second complex that can influence cell division and cell death has revised

the hypothesized relationship between mTOR, growth regulation, and cancer and generated many new avenues for speculation and exploration by cancer biologists and the pharmaceutical industry.

The second mTOR complex, called mTORC2, also contains mLST8, but instead of Raptor, this complex contains the Rictor and mSin1 proteins (Figure 12-1; 17). Understanding the function of mTORC2 initially eluded researchers because the complex is insensitive to acute rapamycin treatment and thus rapamycin could not be used to probe for mTORC2 substrates. However, advances in RNA-interference technology allowed the specific depletion of Rictor from cells leading to the discovery that mTOR, when outfitted with mTORC2-specific regulatory proteins, phosphorylates and activates the Akt/PKB kinase. Current models suggest that upon recruitment to the membrane after growth factor stimulation, Akt/PKB is phosphorylated by mTORC2 with PDK1, and this coregulation is presumed necessary for full Akt/PKB activation. How mTORC2 activity itself is controlled is still being worked out, but initial experiments indicate that growth factors also modulate mTORC2 activity by an unknown mechanism.

As discussed previously, Akt/PKB has a role in cell growth control, but historically it is more recognized for its proposed roles in cell proliferation, cell survival, and metabolism. Thus by coupling mTOR activity with proliferation and survival, the discovery of mTORC2, together with the well-known role of mTORC1 in controlling cell size, strengthens the argument that mTOR growth regulation extends beyond cell autonomous growth to organ and organism growth. Moreover, because of the widespread role of PI3K-Akt/PKB signaling in cancer (Table 12-1), the finding that mTORC2 phosphorylates and activates Akt/PKB is a compelling link between mTOR and cancer (7,8). In retrospect, it is not surprising that mTOR can phosphorylate Akt/PKB because the mTORC2 phosphorylation site on Akt/PKB (S473) is structurally similar to the mTORC1 site on S6K1 (T389), both of which are present in carboxy-terminal hydrophobic motifs. The discovery of the mTORC2-Akt/PKB signaling module also sets up the peculiar situation whereby mTOR can regulate itself in a manner dependent on what proteins it interacts with. For instance, mTORC2-mediated phosphorylation of Akt/PKB may promote subsequent phosphorylation and inactivation of TSC2 (as described in the previous section), which would place mTORC2 upstream of mTORC1 regulation. Whether cells rely on such regulation *in vivo* and whether this type of signaling circuitry is important in human cancer remains to be proven.

Controlling Body Size

It is a remarkable feat for mammals to coordinate the development of their organs to appropriate sizes that are proportional to overall body size. The endocrine system is responsible for conducting this massively orchestrated systemic growth. The major hormone responsible for controlling postnatal growth is growth hormone (GH). GH is synthesized in the pituitary gland and following release into the blood, circulating GH stimulates the release of insulin-like growth factors (IGFs) from the liver. IGFs subsequently stimulate bone and muscle growth, which are the two organs most relevant to determining body size.

The findings that (1) mTOR regulates cell growth, proliferation, and survival; and (2) that both mTORC1 and mTORC2 are downstream of insulin/IGF signaling raises an important question: Is mTOR a “master regulator” of body size? The role of mTORC1 as a nutrient-sensitive regulator of eukaryotic cell growth is an ancient function conserved from yeast to humans. During the evolution of multicellular organisms, growth factor signaling may have been grafted onto the mTORC1 pathway to modulate autonomous cell growth in conjunction with other cells and tissues. The discovery that mTORC2 directly regulates Akt/PKB extends the functions of mTOR to additionally include controlling cell proliferation and survival. mTORC2 is also conserved in yeast, although the protein sequences of some components and the downstream functions of the complex may have diverged. Because of its nutrient and growth-factor-sensing capabilities and the realization of its sweeping roles in the cell life cycle, mTOR seems poised to be a master regulator of organ and body size control. Addressing the global role of mTOR in body size control is difficult because mice null for mTOR are early embryonic lethal. However, mice and flies deficient for S6K1 are viable but reduced in size compared with wild-type counterparts because of smaller cells, implying a link between mTORC1 signaling and body size control (2). It seems likely that mTOR has tissue-specific roles, such as in hormone-producing organs, which will impact overall body size. In fact, in *Drosophila*, the fat body (which may be equivalent to the vertebrate liver) functions as a nutrient-sensing organ that can control global growth through a TOR-dependent mechanism (18). Uncovering such functions in mammals will require the development of new tools.

Targeting mTOR Signaling as a Treatment for Cancer and Other Human Diseases of Cell Growth

With a role for mTOR signaling in cancer firmly established, interest in developing molecules that inhibit mTOR for therapeutic purposes has attracted much attention from the pharmaceutical industry. As mentioned earlier, rapamycin (also known as sirolimus) is already used in the clinic as an immunosuppressant and to prevent restenosis. Rapamycin analogues such as CCI-779 (temsirolimus), AP23573, and RAD001 (everolimus) have been launched into clinical development as anticancer drugs (8,9,17). Preclinical reports indicate that rapamycin has promising antitumor effects in cells with PTEN deficiencies. Work in mice substantiates this by demonstrating that aberrantly proliferating cells with abnormally high Akt/PKB activity are sensitive to the drug. However, rapamycin has shown limited success in the clinic against human cancers known to have elevated PI3K-Akt/PKB signaling. Response to rapamycin as a single-agent therapy is highly variable depending on the cancer, with mantle cell lymphoma, renal cell carcinoma, and endometrial cancers showing the most promise. Unfortunately, no clear biomarker, such as PTEN inactivation or S6K1 up-regulation, has emerged as a successful and consistent predictor of rapamycin's effectiveness. In fact, many cancers linked

to PI3K activation or PTEN loss, such as glioblastoma, have low response rates based on the current treatment strategies.

Although initial reports are disappointing, until the reasons why only some cells are sensitive to rapamycin are understood, it is premature to make conclusions regarding its utility as an anticancer drug. For instance, by inhibiting mTORC1, rapamycin could relieve the S6K1 inhibitory feedback loop to the PI3K-Akt/PKB pathway that was discussed earlier. This in effect would boost Akt/PKB signaling, which in turn could drive Akt/PKB-dependent pathways leading to cell survival and proliferation—clearly an undesirable response. Thus using rapamycin in combination with a PI3K-Akt/PKB pathway inhibitor might prove to be better strategy. Moreover, the trials were initiated under the assumption that rapamycin specifically inhibited the mTORC1 growth pathway. A research finding suggests that this may not be the case in all cell types. Studies suggest that prolonged rapamycin treatment can suppress mTOR-dependent phosphorylation of Akt/PKB in some cells by a still puzzling mechanism that may involve inhibition of mTORC2 assembly (19,20). The realization that chronic exposure to rapamycin, which is highly relevant to patient treatment, can inhibit mTORC2 in some cells could explain the drug's effectiveness against certain cancers and may warrant a re-evaluation of treatment strategies. To summarize, it will be critical to dissect the molecular mechanism by which rapamycin functions and identify biomarkers that faithfully predict which cells will respond favorably to the drug.

A variety of other tumor-prone syndromes are linked to mutations that impinge upon mTOR signaling (Table 12-1; 3,5,7–9). These include tuberous sclerosis, caused by mutations in either of the tuberous sclerosis complex (TSC) genes, *TSC1* and *TSC2*, described earlier, a related disease, lymphangioleiomyomatosis (LAM; *TSC1* and *TSC2*), Cowden disease (*PTEN*), Peutz-Jeghers syndrome (*LKB1*), and neurofibromatosis (*NF1*). A hallmark feature of these diseases is the occurrence of tumor-like growths called hamartomas, which are composed of abnormal tissue elements in abnormal amounts derived from the tissue of origin. Patients suffering from these syndromes are also candidates for therapeutic intervention with rapamycin.

Tuberous sclerosis is characterized by benign tumors that can grow in the brain, kidney, heart, eyes, lungs, and skin and can be devastating and fatal depending on the degree of penetrance and location of the tumors. LAM results in abnormal growth of smooth muscle cells in the lungs, usually affecting women, and can severely compromise respiration. The discovery that the TSC1/2 complex negatively regulates mTORC1 rapidly propelled rapamycin into clinical trials to treat patients suffering from these diseases. Early reports suggest that some features of tuberous sclerosis, such as the appearance of abnormally large astrocytomas in the brain, can be reduced in size following rapamycin treatment. However, TSC1/2 and its target, Rheb, are thought to have mTORC1-independent functions and some studies have concluded that not all cellular phenotypes associated with losing TSC1/2 function are rapamycin-sensitive. Although the rationale to use rapamycin to treat tuberous sclerosis and LAM is compelling, it will be important to consider the mTOR-independent roles of TSC1/2 when evaluating trial results.

Inactivation of PTEN, in addition to its strong links to aggressive cancers, can cause Cowden disease. Cowden disease is characterized by hamartomas present in the skin, mucosa, gastrointestinal tract, bones, central nervous system, eyes, and genitourinary tract. Patients are also at a high risk for breast and thyroid carcinomas. As described earlier, PTEN negatively regulates the PI3K-Akt/PKB signaling pathway, which can subsequently inactivate TSC1/2. Peutz-Jeghers syndrome, which is caused by inactivation of the *LKB1* tumor suppressor, results in intestinal hamartomatous polyps and increased risk of intestinal cancer. *LKB1* is a protein kinase that phosphorylates and activates AMPK. As discussed earlier, AMPK inactivates mTORC1 following energy depletion by promoting TSC1/2 function. Neurofibromatosis primarily results in tumors growing on nerve tissue, but can also cause skin and bone abnormalities. The disease results from inactivation of NF1, a Ras-GTPase-activating protein, which when absent, renders phosphorylation of TSC2 by Akt/PKB and S6K1 by mTORC1 resistant to growth factor (but not nutrient) withdrawal. Since loss of NF1 leads to accumulation of Ras in the active GTP-bound state, this may provide the first clues that Ras, a well-known oncogene, may also affect mTORC1 activity.

The links between signaling networks that control cell, organ, and organism growth through mTOR and the onset of human cancer suggests therapeutic interventions targeting this pathway and its regulators could have great promise in the clinic. The potential for rapamycin and its analogues in treating cancer and other growth diseases has already captivated the pharmaceutical industry and substantial investments in its therapeutic potential are driving it through clinical development. However, the mechanism of rapamycin-dependent inhibition of mTOR is still

enigmatic, and effective use of the drug will require an understanding of which cells are sensitive and why. Considerations previously discussed include understanding if and when rapamycin relieves the S6K1-driven negative feedback inhibition on PI3K-Akt/PKB signaling and the consequences of rapamycin-dependent inhibition of autophagy. Combination therapies may be more effective, such as combining rapamycin with a PI3K pathway inhibitor. The development of biomarkers will be critical for predicting which cancers are susceptible to rapamycin and which combination therapies should be used.

With the realization that mTOR exists in two complexes possessing differential sensitivity to rapamycin, there is strong rationale for developing novel inhibitors that target both complexes. As discussed, rapamycin may fulfill that prescription for some cancer cells, but a universal mTOR kinase inhibitor could have more widespread potential. Obtaining structural knowledge of each complex will be a key step to developing such a drug. With any essential molecule, finding an inhibitor with an acceptable therapeutic window and toxicity will be challenging. Since unique interacting proteins define mTORC1 and mTORC2, the potential also exists to develop complex-specific inhibitors that could be used in combination therapy.

The realization that activating mTOR is a vital step in tumorigenesis has provided many new potential avenues for drug development. While rapamycin analogues will probably be the first mTOR inhibitors to hit the market, this is likely only the first class of such drugs. Advances in our understanding of how rapamycin works, and the development of novel, more specific, mTOR inhibitors, will undoubtedly impact the care of patients with cancer.

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Regulation of the Cell Cycle

Basic Principles of Cell Cycle Progression

The essential function of cell cycle control is the regulated duplication of the cells' genetic blueprint and the division of this genetic material such that one copy is provided to each daughter cell following division. The cell cycle can be divided conceptually into four individual phases. The "business" phases include *S phase* or synthesis phase, which is the period during which DNA is replicated and mitosis (*M phase*), where DNA is packaged, the cells divide and DNA is distributed to daughter cells. *S phase* and *M phase* are separated by Gap phases (*G phase*) to provide the cell with a proofreading period to ensure DNA replication is completed and packaged appropriately prior to division. Separating *M phase* from *S phase* is the first Gap phase (*G₁ phase*) and separating *S phase* from *M phase* is the second Gap phase (*G₂ phase*). *G₀* or *quiescence* occurs when cells exit the cell cycle due to the absence of growth-promoting signals or the presence of prodifferentiation signals. Ordered progression through each phase is intricately regulated through both positive and negative regulatory signaling molecules and is the basis of normal organismal development. The consequences of deregulated growth control include failed or altered development and/or neoplastic/cancerous growth. Over the last two decades, a detailed picture of the major regulators of cell cycle control in both model organisms and higher eukaryotes has evolved. In this chapter, we describe the major regulators of cell division control and introduce current concepts regarding their participation in cell growth.

The Cyclin-Dependent Kinases

Cell cycle progression is positively regulated by a family of protein kinases referred to as the cyclin-dependent kinases (CDKs). In yeast, the organism wherein early groundbreaking work defined many major cell cycle regulators, a single CDK regulates cell division: CDC2 (*Saccharomyces pombe*—fission yeast) and CDC28 (*Saccharomyces cerevisiae*—budding yeast). In contrast, multicellular organisms use a distinct CDK whose activity promotes transition through each cell cycle phase (Figure 13-1). CDKs are binary enzymes. The catalytic subunit, the CDK, coordinates adenosine

triphosphate (ATP) and transfers phosphate to appropriate substrates. As a monomer, the CDK has no enzymatic activity; activation requires association with a specific allosteric activator called a *cyclin*. CDK subunits associate with specific cyclins (Table 13-1) during distinct phases of the cell cycle and as active protein kinases trigger transition through cell cycle phases. Although some CDKs can form complexes with multiple cyclins, in most cases active complexes rely on specific partnerships.

Homology among CDKs, at the level of primary amino acid sequence, is in the range of 30% to 40%. The most highly conserved sequence, which contributes directly to cyclin binding, is the PSTAIRE sequence (CDK1, CDK2) or PV/ISTVRE (CDK4, CDK6) where letters refer to individual amino acids comprising this sequence (e.g., P = proline; 1).

Cyclins associate with the CDK subunit through a conserved domain within the cyclin called the cyclin box. The crystal structure of cyclins has revealed that the cyclin box comprises two sets of five α helices that share little primary homology, but share significant homology with respect to structural and folding topology (2). Sequences N- and C-terminal to the cyclin box share little if any homology and contribute to substrate specific interactions and to post-translational regulation of cyclin protein accumulation (e.g., protein degradation).

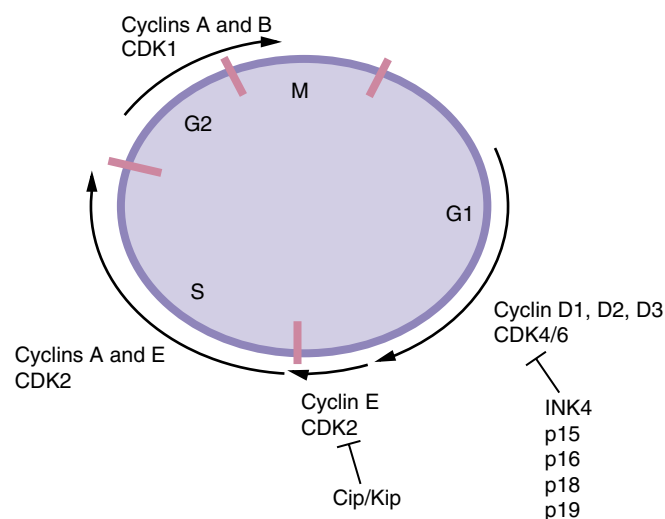


FIGURE 13-1 THE CELL CYCLE.

Table 13-1 CDKs, Activating Cyclins, and Select Substrates

CDK	Cyclin Partner	Substrate
CDK1 (CDC2)	A and B	lamins, histone H1
CDK2	E and A	Rb, p107, p130 Cdt1. CP110
CDK3	C	Rb
CDK4	D	Rb, p107, p130, SMAD2 and 3
CDK6	D	Rb, p107, p130SMAD2 and 3
CDK7 (CAK)	H	CDK1–6, RNA pol II

CDK, cyclin-dependent kinase.

Post-Translational Regulation of CDKs

Regulation of CDKs by Phosphorylation

Cyclin binding to the CDK contributes to kinase activation by inducing a conformational change wherein a C-terminal domain of the CDK, referred to as the “T-loop”, is directed out of the substrate binding cleft (3). In the absence of cyclin binding, the T loop occludes substrate interactions. The cyclin-induced alteration, however, is not sufficient for complete CDK activation. T-loop displacement is ensured by direct phosphorylation of a conserved threonine residue within the T loop (Thr161, CDK1; Thr160, CDK2; Thr172 CDK4) by the CDK activating kinase, CAK (Figure 13-2). In mammalian cells, CAK itself is a cyclin-dependent kinase composed of CDK7 and cyclin H (4). CAK is constitutively active and contributes to CDK activation following cyclin binding via phosphorylation of the T-loop threonine.

Strikingly, CAK (CDK7/cyclin H) not only contributes to CDK activation but is also implicated in transcriptional regulation. Shortly after the identification of CDK7/cyclin H as CAK, CDK7/cyclin H was shown to be the previously identified activity referred to as “TFIIH” (5). TFIIH phosphorylates multiple serine/threonine residues located in the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII), thereby contributing to increased transcriptional initiation (5,6). CDK7 is also conserved in budding yeast. However, in yeast, CDK7 does not contribute to CDK activation. Rather, it is solely a regulator of RNA polymerase activity. Bona fide CAK activity in yeast is contributed by a distinct protein, CAK1 (7,8).

CDK phosphorylation is not solely an activating event. Phosphorylation of N-terminal threonine and tyrosine residues near the ATP binding pocket is inhibitory. Phosphorylation of threonine 14/tyrosine15 is catalyzed by two enzymes, Wee1 and Myt1 (Figure 13-2). Although Wee1 is a cytosolic enzyme, Myt1 is localized to endoplasmic reticulum structures (9). The significance of the differential localization of Wee1 versus Myt1 remains to be established. Threonine 14/tyrosine 15 is located adjacent to the ATP binding pocket of CDKs, providing a structural basis for how phosphorylation of these residues prevents ATP binding (10). Both threonine and tyrosine residues are conserved in CDK1–3, but only the tyrosine residue is present in CDK4–6. Although

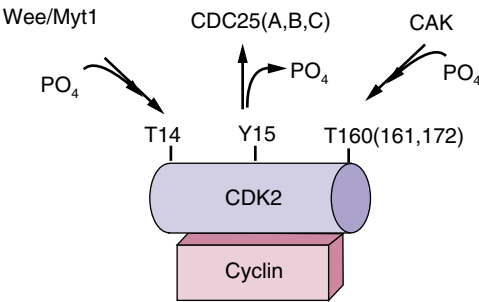


FIGURE 13-2 REGULATION OF CYCLIN-DEPENDENT KINASE (CDK). CDKs are binary enzymes composed of a catalytic subunit, CDK, and a regulatory subunit, cyclin. Activation requires phosphorylation of a C-terminal threonine by the CDK-activating enzyme, CAK. In contrast, phosphorylation of N-terminal threonine, tyrosine residues inhibits adenosine triphosphate (ATP) binding and thus, CDK activity.

phosphorylation of CDK1–2 contributes to the timing of their activation during a normal cell cycle, the CDK4/6 enzymes appear to be subject to this inhibitory phosphorylation only when cells incur DNA damage (11).

In mammalian cells, the removal of N-terminal inhibitory phosphates is catalyzed by any one of three highly related dual-specificity protein phosphatases: CDC25A, CDC25B, or CDC25C (12). In contrast, yeast cells harbor a single CDC25 isoform that carries out all relevant functions. CDC25 isoforms are expressed in a cell cycle–dependent manner and the A-B-C designation corresponds to their order of expression during the cell cycle. CDC25A is expressed first with its expression peaking at the G₁/S boundary. CDC25B expression follows that of CDC25A, with highest levels detected during S phase. Finally, CDC25C is expressed during late G₂ and mitosis. From this expression pattern, substrate specificity was inferred, with CDC25A targeting the G₁ CDKS (CDK4/6 or CDK2–cyclin E), CDC25B regulating the S-phase CDKs (CDK2–cyclin A), and CDC25C regulating mitotic CDKs (CDK1–cyclin B). Consistent with this hypothesis, inhibition of CDC25A resulted in increased CDK2–cyclin E tyrosine phosphorylation (13). Also consistent with substrate specificity being determined by the timing of expression, CDC25 enzymes do not exhibit any specificity toward distinct CDK substrates in vitro. However, timing of expression is not the sole determinant. Deletion of CDC25B or CDC25C, or even the combined deletion does not impair mouse development or cell proliferation in vitro (14). It appears from this analysis that CDC25A expression is sufficient to drive cell cycle expression.

CDK Regulation by Small-Polypeptide Inhibitors

In addition to CDK regulation via phosphorylation, CDKs are subject to direct regulation by small-polypeptide inhibitory proteins referred to as “CDK Inhibitors,” or CKIs (Figure 13-3; 15). These regulators bind directly to and inactivate CDK–cyclin complexes. There are two families of CKIs that have distinct biochemical activities. The *Ink4* (inhibitors of CDK4) family proteins bind exclusively to G₁ CDKs-CDK4 and CDK6. Binding

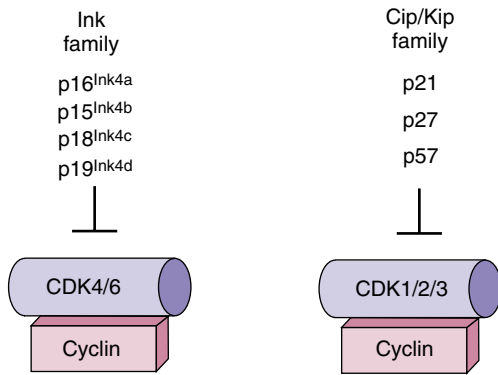


FIGURE 13-3 REGULATION OF CYCLIN-DEPENDENT KINASES (CDKs) BY POLYPEPTIDE INHIBITORS. Two distinct families of CDK inhibitors (CKIs) regulate CDK activity. The Cip/Kip family binds with varying affinities to all CDK/cyclin complexes, but have the greatest inhibitory activity toward CDK2. The Ink4 family (*Inhibitor of CDK4/6*) binds specifically to CDK4/6 and has no capacity to directly regulate other CDKs.

can directly inhibit an active CDK4/6–cyclin complex or Ink4 protein can bind to monomeric CDK4/6 and prevent cyclin association. The second family, *Cip/Kip family proteins*, binds to a broad range of CDK–cyclin complexes, but functionally appear to be negative regulators of CDK2 complexes.

Ink4 Family

The Ink4 family of proteins consists of four members: p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}. All four proteins bind exclusively to and inhibit D-type cyclin-dependent kinases CDK4 and CDK6. The founding member of the Ink4a family was discovered as a protein that interacted with CDK4 in co-immunoprecipitation experiments (16), subsequently identified as MTS1.

Ink4 proteins are homologous in primary structure sharing the presence of four or five ankyrin repeats, which are responsible for protein–protein interactions with CDK4/6. Each repeat consists of an extended strand connected by a *helix-loop-helix* (HLH) motif to the next extended strand. The crystal structure of p19^{Ink4d}–CDK6 complex has been solved and provided valuable details about the mechanism of CDK inhibition by Ink proteins (Figure 13-4; 17). α -Helices and β -turns of p19^{Ink4d} ankyrin repeats form a “cap” over the N-terminal domain of CDK6 and induce its spatial movement away from the C-terminus. This event inhibits productive ATP binding, but does not interfere with the formation of CDK–cyclin complex. As expected from their structure, all four Ink proteins exhibit similar biochemical activities toward CDK4 and CDK6. Interestingly, a short peptide that was derived from one of the ankyrin motifs had the ability to bind and inhibit CDK4, implying the importance of these domains in Ink4 functionality (18).

Despite similar biochemical activities and comparable tertiary structures of Ink proteins, their regulation is distinct. p16^{Ink4a} is not expressed in most tissues. Rather it is induced in response to expression of oncogenic or transforming proteins and during cellular senescence. Several oncogenes as well as tumor suppressors regulate p16^{Ink4a} expression. For example, overexpression of Ras increases p16^{Ink4a} levels in primary rodent cells (19). Inactivation of the retinoblastoma susceptibility protein, Rb, or tumor suppressor

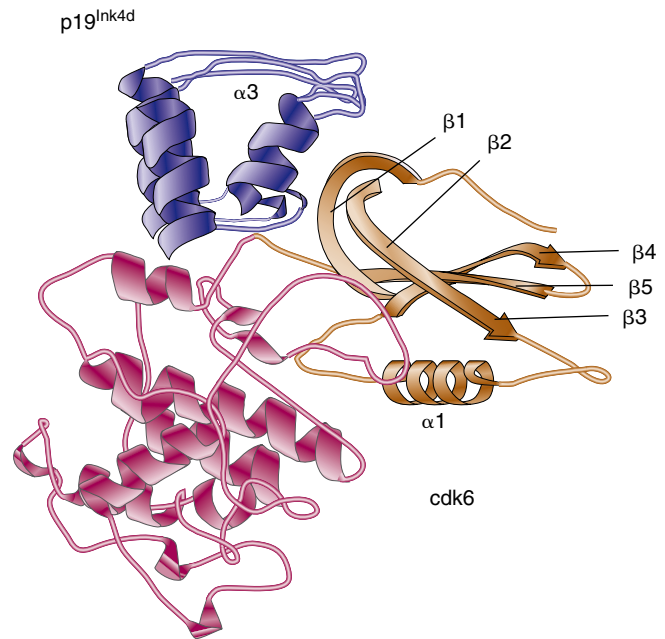


FIGURE 13-4 THREE-DIMENSIONAL STRUCTURE OF THE p19^{Ink4d}/CDK6 COMPLEX. p19^{Ink4d} is dark blue, apart from helix $\alpha 3$, which is light blue. The C-terminal domain of Cdk6 is dark brown, whereas the N-terminal domain, which undergoes extensive movement, is light brown.

p53 can also promote p16^{Ink4a} expression (20). In contrast, p15^{Ink4b} expression is regulated by growth-inhibitory factors (antimitogens) such as TGF- β . Only p18^{Ink4c} and p19^{Ink4d} expression seems to be regulated during various phases of cell cycle with expression peaking during S phase (21). In addition, the expression patterns of Ink4 proteins are also differentially regulated during development.

The structure of the genomic Ink4a locus is unique. Transcription through this locus gives rise to two biochemically distinct proteins, p16^{Ink4a} and p19^{ARF}, as a result of alternative exon utilization (22). Although p16^{Ink4a} regulates CDK4/CDK6 activity thereby indirectly regulating the Rb tumor suppressor, p19^{ARF} regulates the p53 tumor suppressor (23). p19^{ARF} acts by attenuating Mdm2-mediated degradation of p53 and is known as an activator of the p53 pathway. Therefore, loss of p19^{ARF} leads to impairment of p53 signaling. Elimination of Ink4a/ARF genetic locus in mice makes animals highly prone to tumor development (24).

Cip/Kip Family

The Cip/Kip family of CKIs includes three members: p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}. Unlike the Ink4 family of CKIs, Cip/Kip inhibitors bind to and efficiently inhibit various CDKs. Members of the Cip/Kip family are highly homologous and share approximately 50% of their sequences. The amino terminus of both, p21^{Cip1} and p27^{Kip1}, contains an RXL (where X is typically basic) sequence that is responsible for binding to cyclins and is called the cyclin-binding motif, Cy. Cip/Kip inhibitors also contain a domain that is responsible for the binding to CDKs (N-terminal in p21^{Cip1} and p27^{Kip1}; 25). The crystal structure of the p27^{Kip1}/cyclin A/cdk2 complex (Figure 13-5) revealed that p27^{Kip1} binds CDK2 at its

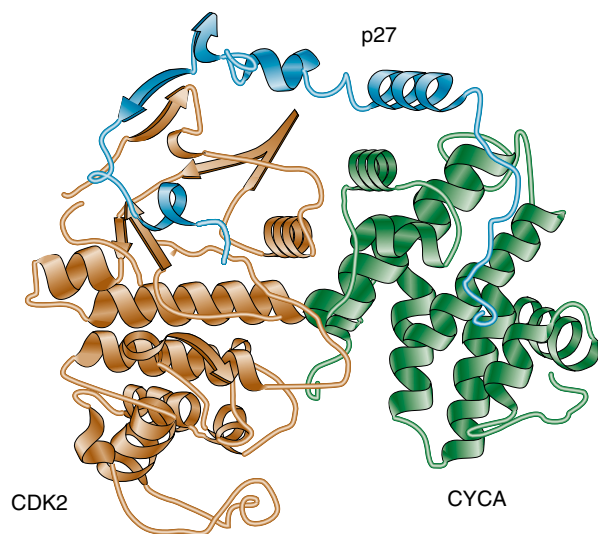


FIGURE 13-5 CYCLIN A/CDK2/p27^{Kip1} COMPLEX. Crystal structure of the inhibited ternary cyclin A/CDK2/p27^{Kip1} complex.

N-terminus and inserts into the catalytic cleft, thus mimicking ATP (26). On cyclin A/CDK2, p27^{Kip1} binds to the groove of the cyclin box. Since both Ink and Cip/Kip proteins occupy almost the same binding sites on CDKs, binding is mutually exclusive. For example, in vitro, p15^{Ink4b} inhibits binding of p27^{Kip1}. However in cells, who gets to the CDK first is often determined by the coordinated cellular localization of the inhibitors and cyclin–CDK complexes.

p27^{Kip1} is responsible for induction and maintenance of the quiescent state. p27^{Kip1} expression is induced in response to growth factor withdrawal and on contact inhibition in cell cultures (27). p27^{Kip1} levels are decreased upon addition of the mitogens by various mechanisms described in subsequent paragraphs. Overexpression of p27^{Kip1} in cells leads to cell cycle arrest in G₁ phase. Unlike p27^{Kip1}, p21^{Cip1} is present at high levels in cycling cells, keeping CDKs activities under tight control. p21^{Cip1} levels are induced in response to DNA damage and genotoxic stress as a result of activation of p53.

Transcriptional Regulation by the E2F Transcription Factors

E2F was originally identified as a cellular DNA binding activity that regulated expression of the viral E2 promoter (28,29). Since this seminal work, molecular analysis has revealed that the E2F activity is encoded by a family of DNA binding proteins, which includes transcriptional activators and repressors. Mammalian cells encode eight known E2F proteins (E2F1–8; Figure 13-6). Further complication ensues from the fact that E2F associates with DNA as a heterodimer; the two known heterodimeric partners for E2F are DP1 and DP2. Indeed the founding member, E2F1 can drive S-phase entry in the absence of growth factor stimulation (30). The ability of E2F1 to drive S phase derives from its role in the regulation of genes whose protein products play essential roles in S-phase progression. Established E2F targets include components

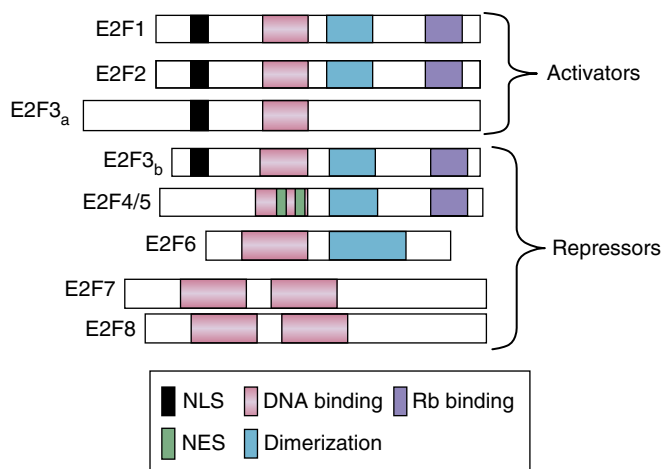


FIGURE 13-6 E2F FAMILY OF TRANSCRIPTION FACTORS. There are eight members of the E2F family of transcription factors. E2Fs are classified as transcriptional activators or repressors. Functional domains are indicated by differential shading.

of DNA replication complexes (MCMs) and S-phase cyclins (E and A; 31). E2F family members were initially considered requisite regulators of S-phase entry. E2F1, E2F2, and E2F3 accumulate during G₁ phase and play critical roles in promoting expression of S-phase targets. Strikingly, E2F4 through E2F7 function as transcriptional repressors (32); E2F3b, an alternatively spliced isoform of E2F3, is also a transcriptional repressor. The E2F repressors function to maintain cells in a quiescent or resting state. In addition to DP1, E2F complexes are further modulated by members of the retinoblastoma protein (pRb) family (pRb, p107, p130; Figure 13-7). The Rb family member functions to recruit chromatin-remodeling enzymes, such as histone deacetylases to E2F complexes. As a consequence, increased activity of E2F1 through E2F3 requires dissociation of “pRb” from the E2F/DP1 heterodimer. As illustrated in the following sections, the G₁ CDK/cyclin kinase triggers this through direct phosphorylation of the associated pRb family member (33).

In addition to the regulation of S-phase entry and progression, E2F transcriptional activators can trigger apoptosis or cell suicide. The mechanisms whereby E2F induces cell death remain unclear. However, pro-apoptotic genes have been identified as E2F target genes. Examples include the p19^{Arf} protein, which is a known regulator of the p53 tumor suppressor. In addition, E2F can increase expression of pro-apoptotic proteins Puma, Noxa, and Bim and repress the anti-apoptotic the Bcl2 family protein, Mcl1.

G₁ Regulation/Restriction Point Control

During the first Gap phase or G₁, cells prepare for DNA replication. They must synthesize proteins necessary to replicate their genome, and once these are made, assemble the various components of the DNA replication machinery on chromatin at so-called origins of replication. This is coordinated with nutrient and growth factor availability to ensure the cell is in an environment that supports cell division. The G₁ phase of the cell cycle is unique in that it represents the only time wherein cells are sensitive to signals from

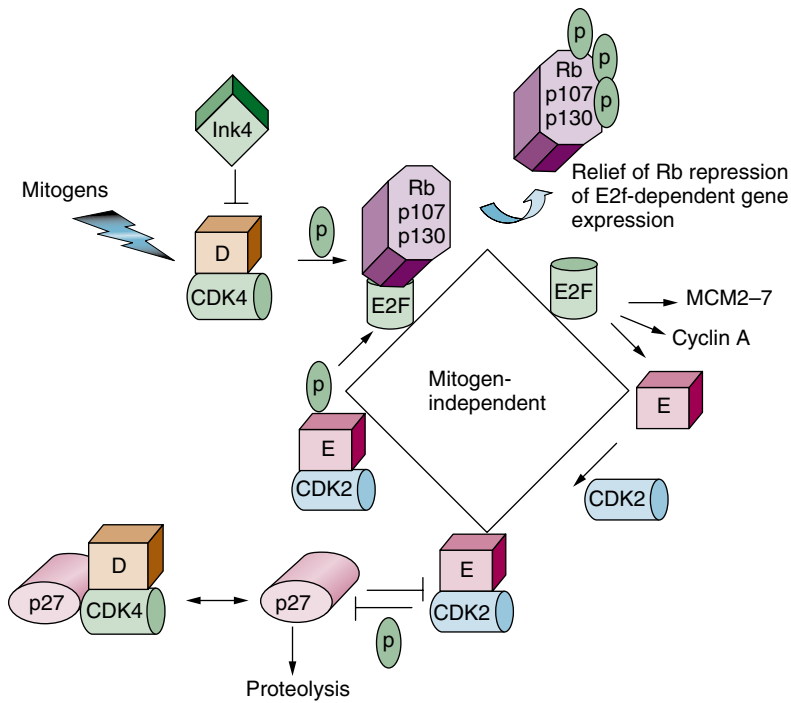


FIGURE 13-7 RESTRICTION POINT CONTROL. Progression through G_1 phase requires growth factor–mediated (mitogenic) signals. Mitogens promote the activation of the initial cyclin-dependent kinase (CDK; cyclin D/CDK4) complex, which phosphorylates Rb family proteins (inactivating signal). The CDK4 enzyme also binds to Cip/Kip CDK inhibitors (CKIs), thereby sequestering these proteins to facilitate CDK2 activation. Rb phosphorylation releases the transcription activating E2Fs (E2F1–E2F3), which promote transcription of downstream targets such as cyclin E, A, and MCM proteins. Cyclin E binds to CDK2, and this active complex maintains Rb in an inactive state. Active cyclin E/CDK2 also targets its own inhibitor (p27^{Kip1}) for proteolysis via site-specific phosphorylation. The complete activation of CDK2, first by cyclin E and then cyclin A, marks passage through the restriction point. Once past this point, cells no longer require growth-factor stimulation for progression through the remainder of that cell division.

their extracellular environment. These signals are in the form of adhesion to substratum and growth factors. Cells require growth-factor-dependent signals up to a point in late G₁ referred to as the restriction point ("start" in yeast).

Progression through G₁ phase is driven by the collective activities of two distinct CDKs. The first is CDK4 or CDK6 in combination with a D-type cyclin. Mammalian cells encode three distinct D cyclins (D1, D2, D3), which are expressed in a tissue-specific manner. Although CDK4 and CDK6 are constitutively expressed, D cyclins are expressed in response to growth-factor signaling. Following accumulation of active cyclin D/CDK4 or CDK6, the CDK2 kinase in combination with cyclin E accumulates to facilitate the transition from G₁ to S phase.

A key protein that regulates G₁-phase progression in the mammalian cell cycle is retinoblastoma protein, Rb. The Rb family consists of three members, Rb, p107, and p130. In quiescent cells, Rb proteins associate with E2F transcription factors to repress E2F-dependent transcription. E2F targets include genes responsible for regulation of cell cycle and DNA replication, such as cyclins E and A (Figure 13-7). Rb activity is regulated at the level of post-translational modification, specifically phosphorylation. Hypophosphorylated Rb is active and binds to E2F thereby silencing E2F-dependent activity. Hypophosphorylated Rb family proteins therefore play a central role in maintaining cells in a resting or quiescent state. Quiescent cells reenter the cell cycle in response to mitogenic growth factors. Growth factor signaling induces the expression of D-type cyclins at transcriptional and post-translational levels (34), leading to activation of cyclin D-dependent kinases CDK4 and -6 and subsequent Rb phosphorylation. Cyclin D/CDK4 or -6 complexes also have a kinase-independent function. They sequester p21^{Cip1} and p27^{Kip1} CKIs from CDK2 kinases and allow activation of basal CDK2/cyclin E kinases which further phosphorylate Rb family proteins. Phosphorylation of Rb promotes

its dissociation from E2F, allowing transcriptional activation of E2F targets such as cyclin E. The E2F-dependent spike in cyclin E, and thus CDK2/cyclin E activity, represents the transition from mitogen-dependent to mitogen-independent cell cycle progression (or passage through the restriction point). In addition to maintaining Rb proteins in a hyperphosphorylated (inactive) state, the activation of cyclin E/CDK2 promotes proteasome-dependent degradation of its own inhibitor p27^{Kip1} (described in a subsequent section). These changes, which include cyclin D/CDK4/6 and cyclin E/CDK2 activation, Rb phosphorylation, and destruction of p27^{Kip1}, render cells with decreased mitogen dependency and are irreversibly committed to enter S phase of cell cycle.

Regulation of DNA Replication (S Phase)

Early experimentation, which relied on techniques wherein two cells (generally one human and one rodent cell) in distinct phases of the cell cycle are fused together (one cytoplasm containing the two distinct nuclei), revealed that chromosomes were competent for duplication in G_1 and S phases. For example, fusing an S-phase cell with a G_1 -phase cell could enforce replication of a G_1 cell; in contrast fusion of a cell in G_2 phase with an S-phase cell could not enforce replication of G_2 chromosomes. It was inferred from these experiments that S-phase cells contained a factor that triggered replication initiation and that G_1 chromosomes were prepared or "licensed" for this initiating activity. Research efforts have shed light on the molecular basis of regulated replication initiation.

While DNA is actively replicated during S phase, cells must prepare DNA for replication during the preceding G₁ phase. During G₁ phase, *origins* (chromatin positions where DNA polymerase complexes initiate replication) must first be established or “licensed.” Licensing refers to the formation of the pre-RC (prereplication

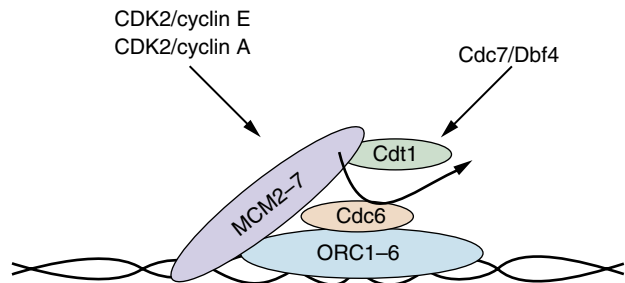


FIGURE 13-8 PREREPLICATION COMPLEX. Prereplication complexes (pre-RCs) form during mid- to late G₁ phase and once formed, origins of replication are considered licensed for replication. Origins are recognized first by the hexameric origin of replication complex (ORC1–ORC6), which serves as a landing pad for recruitment of the remaining components. Following ORC recognition, Cdt1 and CDC6 function in a concerted fashion to recruit the MCM2–MCM7 complex, which is considered the replicative helicase. At the beginning of S phase, additional factors (MCM10, CDC45, and polymerases) are recruited and replication can initiate in a fashion dependent on the CDK2 and CDC7 kinase activities.

complex) at origins of replication (Figure 13-8). Initially, the origin of replication complex ORC must be associated with chromatin to act as a landing pad on which the pre-RC is formed. Unlike most components of the pre-RC, ORC remains constitutively bound to DNA. In budding yeast, ORC acts as a sequence-specific DNA binding complex; however, in fission yeast and mammalian cells no sequence specificity has been elucidated for ORC. The next step is the recruitment of Cdc6 to the ORC. Cdc6 subsequently recruits the MCM complex and Cdt1. However, MCMs are not stably bound at this point. Stable loading of the MCM2–7 helicase complex requires ATP hydrolysis by CDC6, which also results in release of Cdt1 (35). At the G₁/S boundary additional factors are recruited, including MCM10, which functions to recruit Cdc45 and subsequently, DNA polymerase α and primase.

Like G₁ phase, both the G₁/S transition, and S-phase progression are driven by cyclin-dependent kinases (CDK2/cyclin E and CDK2/cyclin A respectively) along with the activity of a distinct CDK-like protein kinase, Cdc7/Dbf4. The precise substrates that must be phosphorylated for the firing of origins remain to be conclusively identified. Substrates identified so far include ORC1, MCM2, and MCM4. Not all origins fire simultaneously, but they are temporally regulated. Origins can be grouped generally into those that initiate at the beginning of S phase, “early,” and those that fire toward the middle to end of S-phase, “late.” The temporal control of firing most likely reflects local controls (chromatin structure modifications) and activation of the complex via phosphorylation.

Paradoxically, although origin firing requires CDK activity, CDK2 activity is also essential for inhibition of a second-round DNA replication (re-replication) within the same cell cycle. While the precise mechanisms whereby CDK2 prevents replication are still under intense investigation, one way it achieves this goal is through direct regulation of Cdt1 levels. On release from the pre-RC, Cdt1 is subject to ubiquitin-mediated proteolysis. Ubiquitination of Cdt1 is in turn facilitated by CDK2-dependent phosphorylation, which targets it to ubiquitinating machinery (36). In addition to Cdt1, MCM complexes dissociate from DNA during replication. Whether this dissociation reflects dislodgment from chromatin by polymerases or also reflects a CDK-dependent function remains to be established (Table 13-2).

Table 13-2 Regulators of DNA Replication and Function

Cdt1	Associates with MCM2–MCM7 and in concert with Cdc6; facilitates MCM loading on origins.
Cdc6	Functions to recruit and load the MCM complex in an ATPase-dependent manner.
Cdc45	Associates with the MCM and is responsible for recruitment of DNA polymerase α , primase, and replication protein A.
MCM2–7	Minichromosome maintenance proteins. Hetero-hexameric complex composed of six distinct but related proteins (MCM2–MCM7). The MCM complex functions as the putative replicative helicase.
MCM10	Structurally distinct from MCM2–MCM7; functions to recruit CDC45.
Orc	Origin recognition complex. Hetero-hexameric complex that binds directly to DNA and functions as a protein landing pad on which the replication complexes form.
Origin	Functionally defined in mammalian cells as regions of chromatin where DNA replication initiates.
Cdc7/Dbf4	The Cdc7 protein kinase, like cyclin-dependent kinases (CDKs) requires an allosteric activator, Dbf4. The Cdc7/Dbf4 kinase phosphorylates components of the replication complexes to initiate DNA replication.
Pre-RC	The prereplication forms during G ₁ and contains ORC1–ORC6, Cdc6, MCM2–7. Replication ensues at S phase on recruitment of DNA polymerase and phosphorylation by both the Cdc7/Dbf4 and CDK2–cyclin A protein kinases.

MCM, mini chromosome maintenance; ORC, origin of replication complex.

G₂/M Transition Regulation

The Kinases of Mitosis

The transition from the second Gap phase (G₂) to mitosis (prophase, metaphase, anaphase, telophase) is regulated by CDK1 (formerly Cdc2) in association primarily with cyclin B (37). Like other CDKs, CDK1 is relatively stable and activation depends first on accumulation of cyclin B. Mitotic cyclins accumulate during S phase and associate with CDK1; however, this complex is maintained in an inactive form via two mechanisms. In the first Wee/Myt1-dependent phosphorylation of Thr-14/Tyr15 prevents ATP binding. The second mechanism relies on active transport of CDK1/cyclin complexes out of the nucleus. Onset of mitosis is triggered by dephosphorylation of CDK1 by a CDC25 isoform and consummate increased nuclear transport/decreased nuclear exit of CDK1/cyclin complexes. Substrates for CDK1/cyclin B include APC20 (a component of the E3 ligase that ultimately degrades cyclin B), microtubule effectors, microtubule motor proteins, and tubulin itself (38). From this and related work, it is clear that CDK1-dependent phosphorylation plays a significant role in the formation and regulation of cellular mitotic structures.

In addition to CDK1, a second family of kinases, called polo-like kinases (PLKs), also contributes to mitotic progression. In mammalian cells, there are four PLKs (PLK1–PLK4) with

PLK1 being the human homolog of the founding member, *Drosophila* polo (39). PLKs are serine/threonine kinases. Structurally, they consist of an N-terminal kinase domain and a C-terminus with one (PLK3) or two (PLK1–PLK3) “polo box” domains. Current models suggest that PLKs are not constitutively active kinases. Rather, PLKs substrates are first phosphorylated by CDKs (e.g., CDK1/cyclin B). Phosphorylation by CDKs is thought to provide a docking site for the polo box domain. Binding of the polo box results in a conformation change in PLKs resulting in its activation where upon it phosphorylates additional critical residues within the substrate. Alternative models suggest that PLKs are activated through phosphorylation by an upstream kinase, such as CDK1/cyclin B. Although CDK1 can indeed phosphorylate PLK1 in vitro, the functional significance of phosphorylation has not been established. Importantly, neither model is mutually exclusive and both regulatory mechanisms could contribute to the regulation of PLK activity in cells.

Like CDKs, substrates for PLKs are still being elucidated. As alluded to in previous sections, many PLK substrates may also be CDK substrates. Substrates of PLK1 include CDC25C and Wee1. The consequence of PLK phosphorylation depends on the substrate. Whereas PLK-dependent phosphorylation of CDC25C promotes its activation during mitosis, phosphorylation of Wee1 promotes Wee1 destruction.

Entry into Mitosis

Entry into mitosis requires the nuclear accumulation of active CDK1/cyclin B kinase. During interphase, activity is low. During G_2 , cyclin B accumulates as a consequence of increased gene expression and decreased protein degradation. Newly accumulated cyclin B is free to associate with CDK1. However, these complexes are maintained in the cytoplasm and are inactive as a consequence of the combined activities of Wee1 and Myt1. Activation of CDK1/cyclin B at the G_2/M boundary is triggered through CAK-dependent phosphorylation of Thr161 in the T loop and dephosphorylation of Thr14/Tyr15 by CDC25. The initial dephosphorylation is likely catalyzed by CDC25B. The activated CDK1/cyclin B then targets CDC25C and Wee1 to promote CDC25C activity and Wee1 destruction, respectively, thereby forming an amplification loop that drives mitotic progression. The accumulation of CDK1/cyclin B in the nucleus is facilitated by phosphorylation of cyclin B near its nuclear export signal, which thereby impedes nuclear exit. PLK1 contributes to mitotic entry and progression by facilitating

these processes. PLK1 can phosphorylate cyclin B just outside the NES (serine 133), thereby preventing nuclear exit. Like the CDK1/cyclin B kinase, PLK1 can also phosphorylate both CDC25C and Wee1 again contributing to CDC25C activation and Wee1 destruction and thereby ensure full CDK1/cyclin B activation.

Chromosome Cohesion

G_2 phase and the beginning of mitosis are denoted by a 4-N DNA content. Following DNA replication and prior to cell division (cytokinesis), cells must maintain the integrity and proximity of the recently duplicated chromosomes (sister chromatids). Prior to segregation, sister chromatids are held together or “glued” by a multiprotein complex called cohesin (40,41). The cohesin complex ensures that sister chromatids are recognized and properly aligned during metaphase. Once aligned, segregation ensues following proteolytic cleavage of cohesin components. Cohesin is composed of four subunits, Smc1/3 and Scc1/3. Smc1 and Smc3 heterodimerize in a head-to-head, tail-to-tail fashion to form ring structure in an ATP-dependent manner. The Scc1/3 subunits associate with the Smc heads to complete the structure (Figure 13-9). The Scc1 subunit contacts both Smc1 and 3 and likely stabilizes the ring structure. Models suggest that the cohesin ring has a diameter of approximately 50 nM; sufficiently large to encircle two sister chromatids (42). Cohesin is envisioned to function by binding and encircling DNA thereby “gluing” sister chromatids together until released.

Exit from Mitosis

During mitotic prophase, chromosome structures are again altered by a complex called condensin, which serves to package chromosomes prior to mitotic division (43). The mitotic spindle also forms during prophase. The mitotic spindle is a bilaterally symmetric, microtubule organizing center shaped like a football. Each half of the spindle contains a *centrosome* and three distinct sets of microtubules (astral, kinetochore, and polar); the kinetochore microtubules are those that attach to chromosomes at the kinetochores to facilitate movement to opposite poles prior to cytokinesis. PLKs are also implicated in the formation of mitotic spindles (44). Loss-of-function experiments in multiple organisms (yeast to mammalian cells) result in the formation of monopolar spindles. During metaphase, the chromosomes align along the “metaphase plate” in preparation for cell division. Anaphase is marked

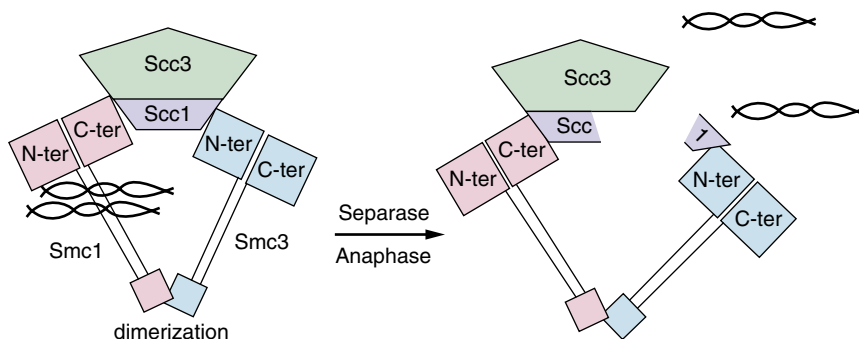


FIGURE 13-9 CHROMOSOMES ARE HELD TOGETHER BY A COMPLEX CALLED COHESIN. Smc1 and Smc3 form a protein ring that held together by a dimerization “hinge” region that encircles chromatids. The Scc1 and Scc3 subunits interact with the Smc “heads,” which retain intrinsic ATPase activity essential for separation of heads to allow DNA to enter. Once all chromatids are aligned during mitosis, Scc1 is cleaved by a protease called Separase to open the ring and allow movement to opposite spindles.

by segregation of chromosomes to opposite poles. The proteolytic cleavage of the Scc1 protein by a protease called *separase* triggers the opening of the cohesin ring thereby allowing chromosome segregation. Anaphase is also marked by the loss of CDK1 activity, which results from proteolytic destruction of cyclin B and cyclin A. The loss of cohesin and mitotic cyclins is coordinated by a multisubunit E3 ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C; see subsequent sections).

Mitotic Checkpoint

The primary goal of mitosis is to ensure that each daughter cell receives one chromosome compliment after cellular division. During mitosis it means that a cell divides only after chromosomes are attached to the microtubules of the mitotic spindle. The *mitotic checkpoint*, or *spindle assembly checkpoint*, is activated as cells enter mitosis, in prometaphase, where it is triggered by *unattached kinetochores* leading to the delay of anaphase onset. Thus, the role of the proteins that are involved in mitotic checkpoint signaling is to sense the attachment and/or tension at kinetochores (45). These proteins are often found to be kinetochore-associated and comprise the *mitotic checkpoint complex* (MCC). MCC includes BubR1 and Mps1 kinases, CENP-E (*centromere protein E*), Mad (*mitotic arrest deficiency proteins*)–1 and -2, and others. The mission of mitotic checkpoint kinases is to signal regulatory proteins to inhibit the entry to anaphase. Models suggest that unattached kinetichores lead to phosphorylation of Mad1/2 proteins, which are then directed to the APC/C resulting in the inhibition of its ubiquitin ligating activity. This action ensures that chromosomes are accurately distributed to daughter cells. In human neoplasia, the activity of mitotic checkpoint can be inactivated through mutations in components of MCC (46), contributing to aberrant mitotic divisions and appearance of aneuploid cells (genetic instability).

Regulated Proteolysis in Cell Cycle Control

Levels of cyclins and CKIs are tightly regulated throughout the cell cycle. This degree of regulation is achieved by coupling the rate of gene expression with regulated proteolysis, which occurs through the ubiquitin proteasome system. The ubiquitin polypeptide consists of 76 residues and is covalently attached to proteins destined for degradation. Attachment occurs through a reversible isopeptide linkage between the carboxyl-terminus of ubiquitin and lysine residue in the sequence of protein. The name *ubiquitin* derives from early observations of its ubiquitous expression. Indeed, ubiquitin is a highly conserved protein throughout evolution from yeast to humans.

Modification of proteins (*ubiquitylation*) with ubiquitin polypeptides requires a conserved series of enzymes. This system includes the ubiquitin-activating enzyme (E1) that performs ATP-dependent activation of ubiquitin. There is only one known E1 enzyme encoded in the human genome. The E1 passes activated ubiquitin to the ubiquitin-conjugating enzyme (E2), of which there are more than 30 (47). In the final stage of ubiquitination, the E2 acts together with an E3, ubiquitin ligase, to attach

mono- or polyubiquitin chains onto the target protein. The E3 ligase acts as the specificity factor that determines substrate recognition and thus comprises the largest group. Once a substrate is polyubiquitylated (four or more tandem ubiquitin molecules on a single lysine within the substrate) it is targeted to the 26S proteasome for degradation.

There are two primary E3 ubiquitin ligases involved in the cell cycle and regulate key cell cycle proteins such as cyclins and CKIs. Both sets of ligases belong to broader E3 subfamily and are called Skp1–Cul1–F-box (SCF) protein ubiquitin ligases and the APC/C. These two systems are structurally similar. However, as one would expect, they target distinct substrates in a cell cycle specific manner and are differentially regulated.

SCF Ligases

The SCF complex consists of variable and invariable components. The core components employed by all SCF ligases include a scaffold protein Cul1; a ring-finger protein, Rbx1/Roc1; and adaptor protein Skp1 (Figure 13-10). The variable component of the SCF ligase, that determines substrate specificity, is the F-box protein (FBP). FBPs bind Skp1 through an F-box motif initially identified in cyclin F and the substrate bringing the two within close proximity. There are approximately 70 F-box proteins reported in mammals (48). F-box proteins are classified accordingly to various protein–protein interaction domains that they use to bind to substrates. WD40 repeats give the name to the FBW class of F-box proteins—leucine-rich repeats (LRRs)—to FBL class and F-box proteins that recognize the substrates through other/unknown protein interaction domains belong to the FBXO (F-box only) class. Structurally, FBPs are organized in a fashion that allows them to recognize diverse substrates. Although substrate recognition by FBPs is generally regulated by phosphorylation of the substrate, recognition by one FBP, FBL2, is determined at least in part by substrate modification with sugar moieties

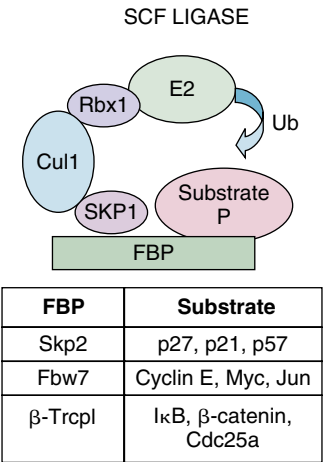


FIGURE 13-10 THE SKP1–CUL1–F-BOX (SCF) E3 LIGASE. F-box protein, or FBP, acts as a specificity component of SCF E3 ligase that recognizes mostly phosphorylated substrates. Further assembly of SKP1–Cul1–Rbx1 components of SCF complex brings E2 ligases and substrates in close proximity for further ubiquitylation. Examples of FBPs and their substrates are indicated in the table.

(N-glycans; 49). Thus, the activity of SCF seems to be constant, but the ability to bind to the target protein is regulated.

One of the most rigorously studied FBPs that is involved in cell cycle regulation is Skp2. Although discovered as cyclin A-associated protein, it has since been implicated in the degradation of CKIs: p27, p21, and p57^{Kip2}. Skp2 knock-out in mice consistent with p27^{Kip1} as bona fide target for Skp2-mediated degradation, since these mice exhibited striking p27^{Kip1} accumulation (50). The binding of Skp2 to p27^{Kip1} requires the phosphorylation of Thr187 by cyclin E/A/CDK2 in p27^{Kip1}. This binding occurs with high affinity only in the presence of another protein, called Cks1 (51). On binding of SCF^{Skp2/Cks1}, phosphorylated p27^{Kip1} is ubiquitinated and undergoes proteasome-dependent degradation in late G₁ and early S phases of the cell cycle. Fbw7, another FBP that has been implicated in the degradation of cell cycle key molecules, targets cyclin E, Myc, and c-Jun for degradation (52). SCF complexes generally regulate proteins involved in G₁ to late S phase, at which point the APC/C is activated and regulates M-phase activities.

APC/C Ligase

Structurally the APC/C ligase is similar to the SCF complex. The core components are Rbx1/Roc1-related ring-finger protein, APC11, a Cul1-related scaffold protein, APC2, and 11 additional proteins with required but essentially unknown functions (53). Two components determine substrate specificity similar to SCF FBPs function: cell division cycle 20 (Cdc20) and Cdh1 (Figure 13-11). APC/C ligases recognize specific sequences in target proteins called the destruction box (D-box) and the Ken box. These short-peptide sequences are recognized by the Cdh1 and Cdc20 specificity adaptors and therefore facilitate recruitment of the active APC/C.

APC/C is active from anaphase through early G₁ phase. However, the regulation of APC/C activity is distinct from SCF ligases. The Cdc20 subunit of APC/C, APC/C^{Cdc20}, itself undergoes activating phosphorylation events by CDK1/cyclin B. APC/C^{Cdc20} can also be phosphorylated and activated by PLK1 and inactivated by PKA. The activity of APC/C^{Cdc20} is regulated by protein–protein interactions. Mitotic spindle checkpoint proteins Mad1/Mad2 bind to and inhibit APC/C^{Cdc20} function, thereby

delaying the onset of anaphase. The substrates of APC/C^{Cdc20} ligase include securin, a protein associated with the mitotic protease separase that allows sister chromatid separation, cyclins A and B. When cyclin B is degraded, CDK1 activity declines, contributing to the activation of APC/C^{Cdh1}; active APC/C^{Cdh1} proceeds to fully ubiquitinate cyclin B molecules, eliminating CDK1 activity. The switch of Cdc20 specificity component of APC/C complex to Cdh1 in late M phase also leads to degradation of Cdc20 itself, Plk1, Aurora A/B kinases, and others (reviewed in [54]). APC/C^{Cdh1} remains active during early G₁ phase where it also ubiquitinates Skp2 permitting p27^{Kip1} and p21^{Cip1} accumulation, as described above.

Integration of Growth-Factor Signals During G₁ Phase by the Ras small GTP-Binding Protein

Growth-factor-dependent signaling promotes the expression and accumulation of factors essential for cell growth (mass accumulation), cell survival, and cell cycle progression. With regard to the cell cycle, growth factor signaling converges on G₁-phase components. Entry to and progression through G₁ phase of the cell cycle requires activation of signal transduction pathways via extracellular growth factors. G₁ progression requires G₁ CDK/cyclin complexes to accumulate and become activated and conversely that CKIs are destroyed. Although this is accomplished through numerous pathways, the molecular basis for Ras-dependent signals in G₁-phase progression is understood with the greatest detail.

Extracellular growth factors promote the Guanosine triphosphate (GTP) loading of Ras, its active form. Active Ras-GTP intersects with the cell cycle via the regulation of cyclin D1 expression and activation of the CDK4/6 kinase (Figure 13-12). Ras-GTP subsequently triggers the activation of multiple independent signaling pathways including canonical MAP kinase signaling Raf, mitogen-activated protein kinase-kinases (MEK1 and –2), and the sustained activation of extracellular signal-regulated protein kinases (ERKs or MAPK). This pathway contributes to cyclin D1 gene expression (55). Ras-GTP triggers the activation of a second related, small-GTP binding protein, Rho; activation of Rho also plays a critical role in growth-factor-dependent cyclin D1 expression during G₁ phase. A third pathway activated by Ras involves PI-3K and Akt (PKB). The activation of this pathway contributes to increased translation of a multitude of proteins, including cyclin D1 by virtue of the ability of Akt to regulate translation initiation (56). Active Akt also inactivates glycogen synthase kinase-3 β (GSK-3 β) by site-specific phosphorylation. Active GSK-3 β kinase phosphorylates cyclin D1, thereby promoting cyclin D1 ubiquitination and proteolysis (57). Thus, inactivation of GSK-3 β is a critical step necessary for cyclin D1 accumulation during G₁ phase.

For cells to progress through G₁ phase, growth-factor signaling must promote increased G₁ cyclin accumulation and suppress accumulation of the cell cycle inhibitor p27^{Kip1}. Active Ras also plays a central role in the regulation of p27^{Kip1} in G₁ phase by decreasing the efficiency of p27^{Kip1} translation and increasing the kinetics of p27^{Kip1} proteolysis. Ras-dependent regulation of p27^{Kip1}

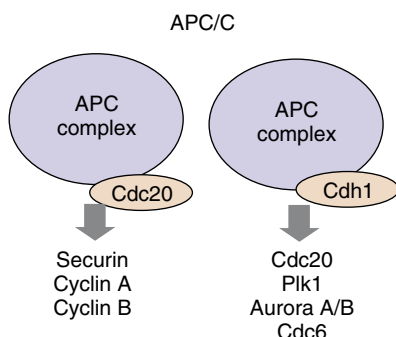
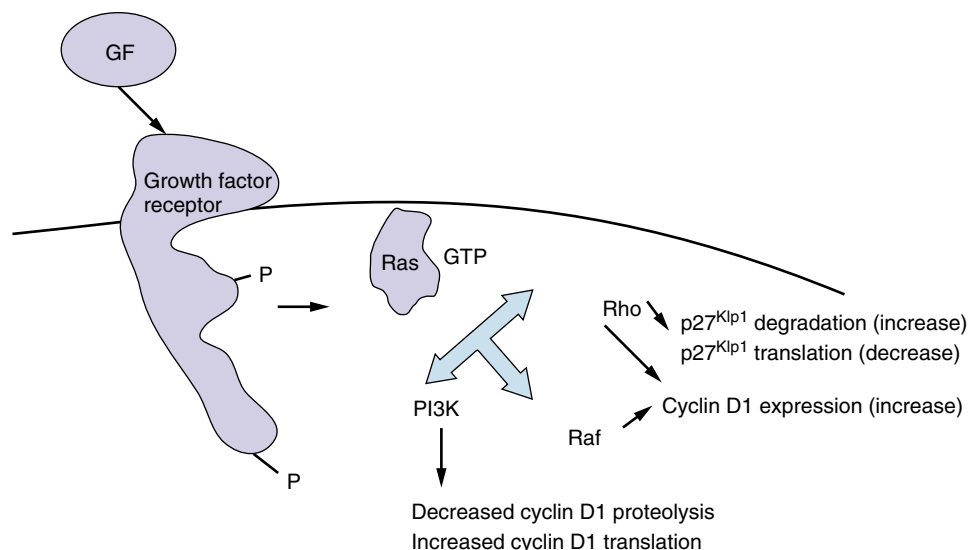


FIGURE 13-11 THE ANAPHASE-PROMOTING COMPLEX/CYCLOSOME (APC/C). APC/C ubiquitin ligase is a multiprotein complex that is active in M through G₁ phases of cell cycle. The subunits that are responsible for the recognition of substrates by APC are Cdc20 and Cdh1.

FIGURE 13-12 MITOGENIC ACTIVATION OF RAS. Ras is generally in a GDP-bound state. Extracellular growth factors promote the exchange of GDP for GTP (GTP-bound Ras or active Ras). Ras-GTP increases signaling through multiple pathways that contribute to increased G_1 cyclin expression and thereby cell cycle progression.



translation and degradation requires Rho signaling. The concerted increase in cyclin D1 accumulation and decrease in p27^{Kip1} accumulation provides a threshold of CDK4/cyclin D1 activity that is necessary and sufficient for restriction point passage and commitment to S-phase entry.

Deregulation of G_1 Restriction Point Control in Cancer

In G_1 phase, cells make the decision to either progress through the restriction point and enter S phase or enter G_0 . These decisions are based on extracellular signals that the cell receives and on the integrity of signaling machinery that detects these signals. Deregulation of G_1 progression is a frequent occurrence in cancer. This can occur through mutations or deregulated expression of CDKs, cyclins, or CKIs. Loss- or gain-of-function mutations in upstream regulators of the CDK kinases also occur in cancer. In this section, we discuss some alterations found in cell cycle regulators in cancer.

Cyclin D-dependent kinases are a primary point of control for the progression through G_1 phase and are linked to cancer progression. Cyclin D1 overexpression is a hallmark of breast and esophageal cancers (58). In many cases this up-regulation is due to cyclin D1 gene amplifications, but can also result from increased transcription (58). In addition to gene expression alterations, decreased cyclin D1 proteolysis is implicated in deregulated cyclin D/CDK4 activity in breast and esophageal cancers. Cyclin D1 overexpression also occurs as a consequence of chromosomal translocations. Amplifications encompassing the *CDK4* and *CDK2* genes have been reported in large B-cell lymphomas, lung tumors, and cervical carcinomas. Downstream targets of cyclin D/CDK4/6 kinases, Rb proteins, are also targeted in cancer. Mutations and deletions in the *Rb* gene are common events in tumors; inactivation of Rb alleviates a cell need for CDK4/6 kinase and thus relieves some cellular dependence on growth factor signals (59).

As one might anticipate, Cip/Kip inhibitors can also function as tumor suppressor proteins in mouse model systems and consistent with this work, their expression is deregulated in human

cancers. p53, the main transcriptional regulator of p21^{Cip1} is often lost or mutated during tumorigenesis. Reduced p27^{Kip1} levels alone or together with increased cyclin E expression are associated with poor prognosis in breast and ovarian carcinomas. Inactivation of p16^{Ink4a} occurs frequently in lung, bladder, and breast carcinomas, as well as leukemia (reviewed in [24]).

In addition to alterations in the expression and integrity of cell cycle genes in cancers, attenuation of their regulatory pathways also occurs. These include signaling pathways (Ras), transcription factors (myc), and components of ubiquitin ligases. Skp2, the specificity component of the SCF ligase for p27^{Kip1}, is up-regulated in variety of tumors, including colon, lung, breast, prostate, and lymphoma (54), where it decreases p27^{Kip1}. Another F-box protein, Fbw7, which regulates degradation of cyclin E, is mutated in ovarian and breast cancers.

Mutations and deregulation of the expression of regulators of mitosis are also observed in human malignancy. Increased accumulation of Cdc20 (APC/C) is observed in lung and gastric tumor cell lines. Mutations in *PLK1* are found in human cancer cell lines and its attenuated expression is observed in colorectal, endometrial, and breast carcinomas.

Conclusion

Significant advances have been made in our understanding of the molecular basis of cell cycle regulation. Conceptually, it was anticipated that understanding the basic mechanisms and regulators would permit scientists to ask how they contribute to organismal development and/or cancer progression. Indeed, these questions are now being addressed through targeted deletion of individual genes in the mouse genome. G_1 cyclins and CDKs have been removed from the mouse genome by targeted deletion to evaluate the role of these molecules in organismal development and basic cell growth. Although each knockout mouse strain has revealed unique properties of each molecule, what has been most striking is the revelation that no one cyclin or CDK is absolutely essential for development (60). Thus, although we have considered each

mammalian CDKs to have distinct substrates, in an intact cell, there is sufficient redundancy to permit loss of any one complex.

The identification of the critical regulators of cell division has also facilitated the development of antiproliferative therapies through design of small-molecule inhibitors of the CDKs. Given that deregulated growth control is a fundamental property of cancer,

the development of small molecules that inhibit the molecular machine that drives cell cycle transitions, is a conceptually attractive therapeutic option. The continued investigation of components of the cell cycle machine will undoubtedly continue to contribute fundamental insights into cell growth control and potentially provide additional insights into diseases that alter growth properties.

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Metabolism of Cell Growth and Proliferation

Why Should Cancer Biologists Care about Metabolism?

The individual cells that make up multicellular organisms depend on instructive signals communicated through cell surface receptors to maintain their survival and to engage in cell division. Cancer results from mutations that promote cell autonomy. Mutations that favor cell autonomous survival and/or proliferation will permit a cell and its progeny to persist and ultimately to accumulate. Perhaps the most fundamental cellular activity is bioenergetic metabolism. Paradoxically, the first studies on tumor cell metabolism suggested that tumors used extracellular nutrients in a particularly wasteful manner. In the 1920s, Otto Warburg observed that tumor cells engaged in a surprisingly high rate of glucose utilization in comparison with normal tissue, a phenomenon now called the Warburg effect (1). Despite the fact that tumor cells retain the same ability to engage in oxidative phosphorylation as nontransformed cells, most tumor cells take up so much glucose that most is secreted as lactate. In modern oncology, the Warburg effect is exploited in positron emission tomography (PET) with radioactive glucose analogs, a useful tool for cancer diagnosis and monitoring (Figure 14-1, inset).

Why do cells from tumors use such a seemingly wasteful metabolic activity? It turns out that this activity is not unique to tumor cells. For a cell to undergo a round of division, all of its components must be duplicated, and this poses a biosynthetic challenge that would be insurmountable without a major transition in nutrient uptake and metabolism by the cell. The “metabolic transformation” indicated by aerobic glycolysis distinguishes quiescent cells with minimal biosynthetic capacity from rapidly proliferating cells in which continuous, robust biosynthesis can be sustained (Figure 14-1). Normally, this metabolic transition is directed by signaling through growth factor receptors. However, it is now evident that many tumor cells possess activating mutations in these signaling pathways, rendering nutrient uptake and utilization cell autonomous. Such a transformation, like loss of cell cycle control or protection from apoptosis, is a common feature of the tumor phenotype, providing a bioenergetic and synthetic platform from which tumors can arise. Our understanding

of how the classical metabolic activities in nonproliferative cells are reprogrammed in the support of cell growth and proliferation is incomplete. The mechanisms by which the genetic changes observed in cancer effect these metabolic changes are considered in the following sections.

Definitions

- **Metabolism** is the sum of the biochemical activities that allow a cell or an organism to maintain bioenergetics and execute tasks. Traditionally, metabolism is concerned with the handling of organic compounds (sugars, amino acids, nucleotides, lipids) through a variety of enzymatic pathways.
- **Metabolic transformation** is a term to describe the collective changes in cellular metabolism that arise from cancer-causing mutations and enable cells to grow and proliferate independently of normal physiologic control mechanisms. The Warburg effect is one component of the metabolic transformation.
- **Bioenergetics** refers to the processes that determine the energy state of a cell. The availability of adenosine triphosphate (ATP) and the ratio of ATP to ADP are important indicators of cellular bioenergetics. Many variables impact bioenergetics, including nutrient availability and the rates of ATP production and consumption.
- **Macromolecules** are the large structural and/or functional components of cells, especially protein, lipid, and nucleic acid. In cancer, a major metabolic consideration is how cells manage to duplicate all their macromolecular constituents to make daughter cells. This involves not only the energy needed to drive macromolecular synthesis (e.g., protein translation), but also the acquisition of substrates like glucose and amino acids, which provide carbon and energy to generate the larger molecules.
- **Growth**, for the purposes of this chapter, is defined as an accumulation of macromolecules (Figure 14-2). **Proliferation** is an increase in the number of cells in a population due to transit through the cell cycle, culminating in cell division. Proliferation usually involves an increase in total biomass of the population.
- **Anabolism** (anabolic metabolism) is the coordinated metabolic activity that allows cells to produce macromolecules. These

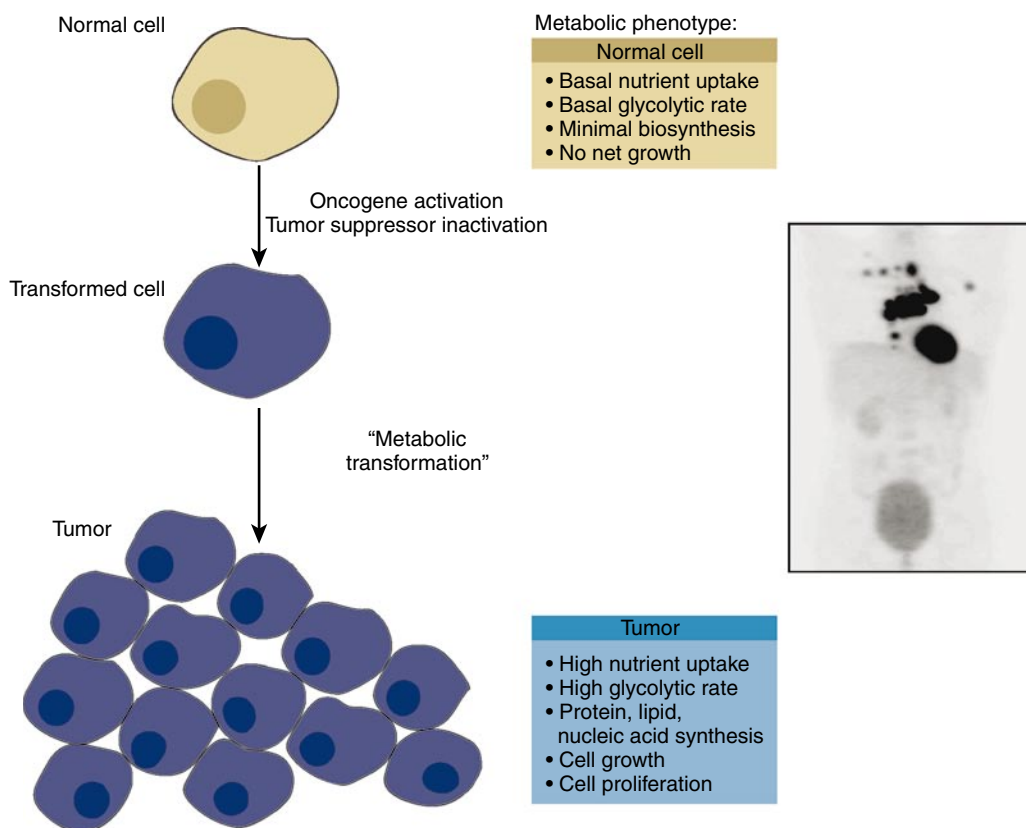


FIGURE 14-1 TUMORS HAVE A METABOLIC PHENOTYPE THAT DISTINGUISHES THEM FROM NORMAL CELLS. Normal, nonproliferating cells are metabolically quiescent, requiring only basal activities to maintain minimal bioenergetic requirements. During tumorigenesis cells acquire mutations that promote cell survival, growth, and proliferation. To develop into a rapidly proliferating tumor, these cells must also undergo a "metabolic transformation" characterized by the myriad activities that support cell growth and proliferation. Therefore, the metabolic transformation is a common feature of aggressive tumors that arise from any of a variety of genetic mechanisms. One characteristic of the metabolic transformation is a robust increase in glucose uptake and phosphorylation compared to normal tissue. This difference can be exploited to identify tumors with [^{18}F] fluorodeoxyglucose–positron emission tomography (FDG-PET) scanning (*inset*).

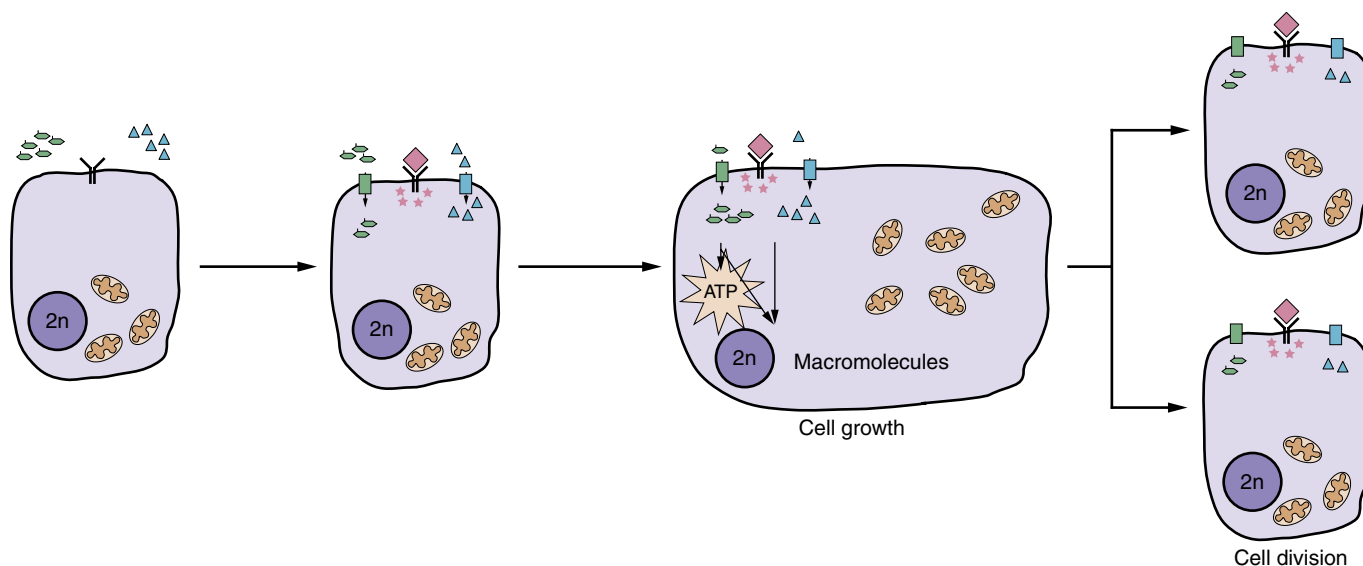


FIGURE 14-2 GROWTH FACTOR SIGNALING STIMULATES CELL GROWTH AND PROLIFERATION. In quiescent cells, uptake of nutrients like glucose (green hexagons) and amino acids (blue triangles) is minimal despite abundance of these substances in the extracellular milieu. When a growth factor (red diamond) binds to its receptor, activation of a signal transduction pathway stimulates the cell to take up nutrients and use them in pathways that generate energy (adenosine triphosphate [ATP]) and support the production of macromolecules needed for cell growth. If the cell enters the cell cycle, the genome is duplicated in S phase and two daughter cells are produced in M phase.

include the pathways for lipid and protein synthesis, both of which are vital to tumorigenesis. In general, anabolic processes consume energy.

- **Catabolism** (catabolic metabolism) is the metabolic activity used to degrade molecules to produce simpler constituents and energy. Examples include β -oxidation of fatty acids and amino acid oxidation, both of which produce ATP at the expense of intermediates that could have otherwise been used for anabolism.

What do Cells Need to Grow and Proliferate?

The metabolism of growth is considerably different from the metabolism used by nonproliferating cells, which need to fulfill various biological roles but are not subject to the biosynthetic challenge of replicative division. This section outlines some of the essential requirements for cell growth to help explain why particular pathways may be enhanced in tumors.

Instruction

Yeast, the simplest eukaryotic organisms, are fully cell autonomous in that they modulate their metabolism, growth, and proliferation on the basis of the availability of nutrients without requiring other extracellular signals (2). When an oxidizable carbon source such as glucose is abundant, yeast increase their ability to use that substrate to maintain a favorable bioenergetic status and engage in metabolic pathways for macromolecular synthesis and proliferation. Elegant nutrient-sensing mechanisms arose in yeast to match metabolic activity with the environment, and these systems enabled yeast to adapt to periods during which some or all carbon sources are scarce. This is critical because yeast have no mechanisms to control the abundance of nutrients in their environment.

By contrast, mammals and other complex eukaryotes have organ systems that are dedicated to maintaining key nutrients like glucose within a narrow range. As a result, mammalian cells are typically not subjected to wide fluctuations in nutrient availability. However, normal mammalian cells do not self-regulate nutrient uptake and utilization. Rather, these activities are coordinated by extracellular growth factors that bind to receptors on the cell surface and stimulate signal-transduction pathways that regulate metabolism and other activities (3), as shown in Figure 14-2. In this fashion, the activities of particular subsets of cells (those with the correct receptor) can be redirected, preparing these cells for orchestrated responses to fulfill the needs of the entire organism. For example, cytokine growth factors instruct cells to proliferate; during an infection, they stimulate growth and proliferation of certain subsets of immune cells needed to contain and clear offending organisms.

The signal-transduction pathways stimulated by growth factors directly elicit changes in nutrient uptake and metabolism, culminating in the engagement of anabolism for growth and

proliferation. An important theme in tumor biology is that growth-factor signal-transduction pathways become constitutively active, driving anabolic metabolism in the absence of cell-extrinsic stimuli. Genetically, positive regulators of growth factor signal transduction pathways behave as oncogenes (activating mutations promote transformation), and negative regulators behave as tumor suppressors (loss-of-function mutations promote transformation).

Substrates for Growth

Passage through the cell cycle results in a doubling of all the macromolecules—protein, lipid and, nucleic acid—that make up a cell. To do this, cells require a large supply of the basic substrates needed for macromolecular synthesis. The most important single substrate is glucose, because intermediates in glucose metabolism contribute directly or indirectly to the synthesis of all three macromolecular classes (Figure 14-3). In addition, protein synthesis requires the essential amino acids, some of which are also used in the synthesis of purines and pyrimidines. Glutamine is particularly important for both biosynthesis and bioenergetics. Proliferation requires other substrates as well, such as the head groups for phospholipid synthesis (choline, ethanolamine) and a variety of inorganic materials, which will not be discussed in depth in this chapter.

Energy and Reducing Equivalents

The energy needed for growth and proliferation far exceeds what cells normally need to perform homeostatic activities. In cancer cells, ATP is generated primarily through glycolysis, which is highly induced in the most rapidly growing tumors. To some extent, oxidative phosphorylation also contributes to ATP production during growth, although anaerobic glucose metabolism appears quantitatively to be the more important process.

Biosynthesis, particularly of nucleic acids and lipids, also requires “reducing equivalents” in the form of reduced electron carriers like NADH and NADPH. The protons and electrons carried by these molecules are transferred to intermediates in synthetic pathways; therefore, they are as important as ATP in some biosynthetic reactions. Reducing equivalents come from a variety of sources, but during growth, a large fraction of both NADH and NADPH is formed through the metabolism of glucose.

Appropriate Regulation of Metabolic Pathways

Most cells have the capacity to perform anabolic or catabolic metabolism. Therefore, a critical issue in growth metabolism is how cells coordinate metabolic activity to maximize biosynthetic efficiency. Cells have many complementary mechanisms to do this. First, flux through metabolic pathways can be controlled by phosphorylation of key rate-controlling enzymes, some of which are targets of growth factor signal transduction. Second, metabolites often exert allosteric effects on metabolic enzymes, increasing flux through desired pathways and suppressing others. Third, increasing evidence suggests that growth factor signaling also regulates

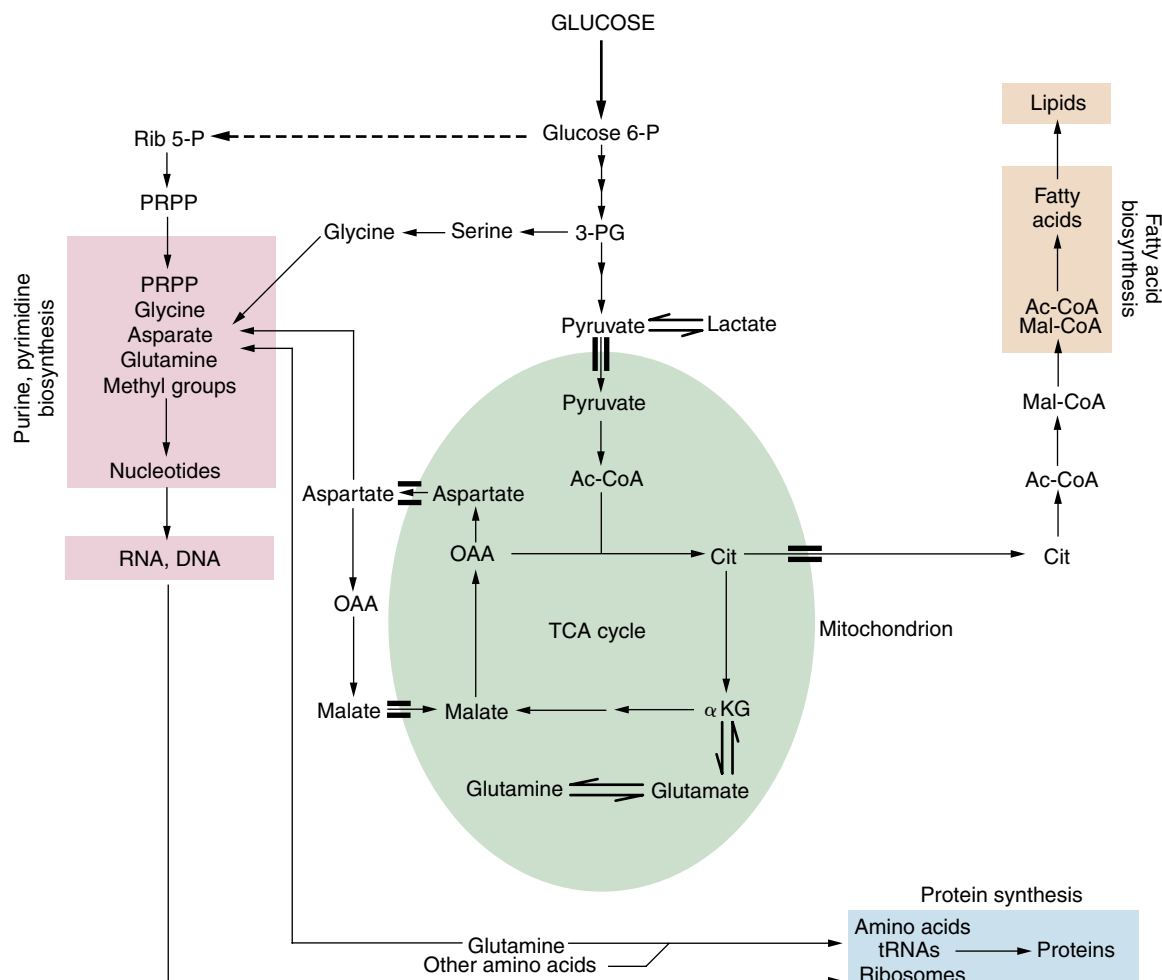


FIGURE 14-3 GLUCOSE SUPPORTS MANY OF THE METABOLIC ACTIVITIES NEEDED FOR CELL GROWTH AND PROLIFERATION. Glucose is rapidly consumed by tumors and is a key metabolite for cell growth and proliferation. The synthesis of all three classes of macromolecules—lipids, proteins, and nucleic acids—use energy and metabolic intermediates generated from glucose. Glycolysis, the main pathway of glucose metabolism, results in formation of pyruvate and lactate. A fraction of pyruvate is imported into the mitochondria, converted to acetyl-CoA and oxidized in the tricarboxylic acid (TCA) cycle. Synthesis of fatty acids and lipids use the TCA intermediate citrate. Transamination of other TCA intermediates produces amino acids like glutamate and aspartate. Protein synthesis requires amino acids as well as the ribosomal machinery, which is composed of protein and nucleic acids. Nucleotide biosynthesis requires input of multiple metabolites as shown; methyl groups are generated by folate metabolism (not shown). 3-PG, 3-phosphoglycerate; α-KG, α-ketoglutarate; Ac-CoA, acetyl-CoA; Cit, citrate; Mal-CoA, malonyl-CoA; OAA, oxaloacetic acid; P, phosphate; PRPP, 5-phosphoribosyl pyrophosphate; Rib 5-P, ribose 5-phosphate.

the abundance of key enzymes through effects on gene expression. This appears to be important in tumors, which often exhibit higher levels of biosynthetic enzymes than normal tissue. The sum of all these effects is to orchestrate the utilization of substrates and the production of energy so as to enable cell growth.

The Metabolic Phenotype of Tumors and Proliferating Cells

Studies on the metabolism of rapidly growing, highly proliferative tumors have documented certain core characteristics that appear to define a characteristic metabolic phenotype. These include (1) a high rate of glucose uptake and glycolysis; (2) a submaximal activity of oxidative metabolism, including the tricarboxylic acid (TCA) cycle; (3) increased glutamine uptake and utilization; and (4) increased production of lipids and nucleic acids. This section highlights these important themes in cancer metabolism by

reviewing the relevant pathways and discussing their function in the growing tumor.

Aerobic Glycolysis: The Warburg Effect

Of all aspects of the metabolic transformation in tumors, the profound enhancement of glucose uptake and glycolysis is the most extensively documented, the most widespread among tumors, and the most clinically useful, both for diagnosis and for monitoring response to chemotherapy. In 1926, Warburg published the seminal observation that rapidly proliferating tumor cells have a remarkably high rate of glucose consumption and lactate production, despite adequate oxygenation to support complete oxidation of glucose to carbon dioxide (CO_2). This phenomenon has been verified by numerous investigators over the subsequent 80 years, so it may seem surprising that there is still controversy regarding how, and why, these cells undergo such high rates of glucose utilization. By examining the glycolytic pathway and the numerous benefits it

provides to growing cells, several clues emerge as to why glucose is so important to tumors.

Glycolysis: Why Is It Good for Growth?

Glucose, at concentrations typically greater than 3 mM, is the most abundant nutrient in mammalian serum. Integrated activities of the liver and pancreas tend to keep glucose levels

within a fairly narrow range, ensuring that cells have near-constant access to it. Glucose import, typically under growth factor control but constitutively activated in many tumors, is an important regulatory step in cellular glucose utilization. In tumors, the major metabolic fate of glucose is to be degraded, primarily through glycolysis, which yields two molecules each of ATP, NADH, and pyruvate (Figure 14-4).

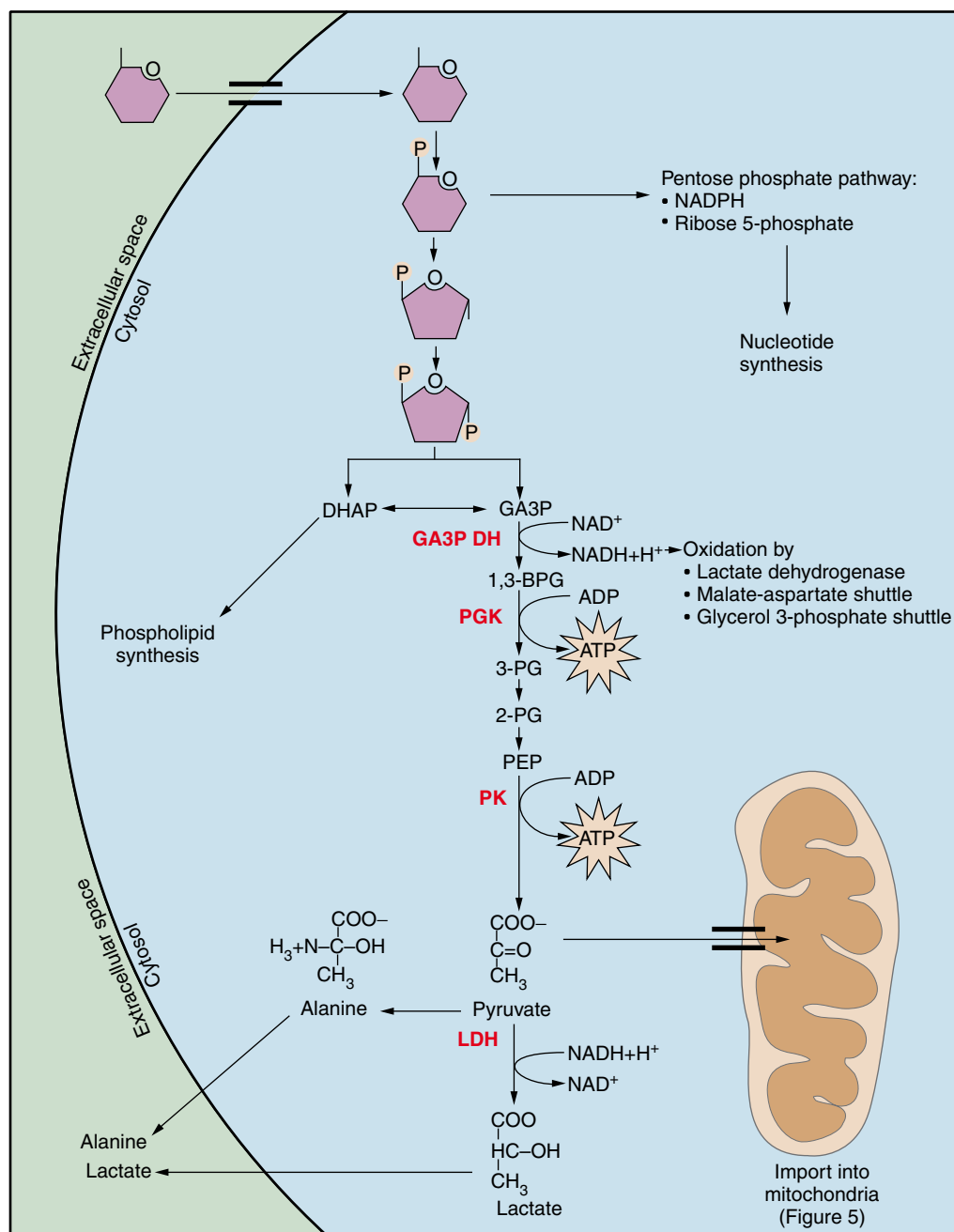


FIGURE 14-4 GLYCOLYSIS PRODUCES ENERGY AND METABOLIC INTERMEDIATES FOR CELL GROWTH. Import and phosphorylation of glucose commits it to further metabolism through the pentose phosphate pathway, which produces NADPH and ribose 5-phosphate for nucleotide synthesis, or glycolysis. Glycolysis generates adenosine triphosphate (ATP) and NADH (a net of two molecules of each, taking into account the two molecules of ATP consumed early in glycolysis), as well as intermediates for phospholipid synthesis and other pathways. The end product pyruvate can be converted to lactate, regenerating NAD⁺; transaminated to alanine; or oxidized further in the mitochondria as shown in Figure 14-5. Metabolic intermediates are in black and enzymes are bolded in red. 1,3-BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; GA3P DH, glyceraldehyde 3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PEP, phosphoenolpyruvate; PGK, phosphoglycerate kinase; PK, pyruvate kinase.

Glucose is an excellent energy source during cell proliferation. First, glycolysis generates cytosolic ATP very rapidly (conversion of glucose to lactate can be observed within seconds), and this would benefit cells with robust biosynthesis of protein, lipid, and nucleotides. Second, the total yield of ATP from glycolysis is actually higher than the two molecules generated by phosphoglycerate kinase (PGK) and pyruvate kinase (PK). This is because a fraction of the NADH generated by glyceraldehyde 3-phosphate dehydrogenase (GA3PDH) is “shuttled” to the mitochondrial electron transport chain for oxidative phosphorylation. The malate-aspartate shuttle and the glycerol 3-phosphate dehydrogenase shuttle accept electrons from NADH and donate them to the electron transport chain, yielding the equivalent of three or two molecules of ATP, respectively, in addition to the ATP synthesized in the cytosol. Numerous studies have documented the activity of these shuttles in cells from tumors (4–7). The complete oxidation of glucose would yield even more ATP (38 molecules per molecule of glucose), but as explained in subsequent sections, tumors do not in general completely oxidize glucose.

In addition to providing energy and reducing equivalents, glucose metabolism provides many of the metabolic intermediates used for cell growth. For example, the glycerol moieties of phospholipids are largely generated from the three-carbon metabolites of glycolysis; lipid synthesis, and consequently cell growth, cannot occur without these. Also, glucose metabolism contributes to nucleotide biosynthesis in several ways. First, glucose flux through the pentose phosphate pathway yields ribose 5–phosphate, a starting point for de novo synthesis of purines and pyrimidines. Second, the pentose phosphate pathway generates NADPH, which provides reducing power for the synthesis of nucleotides and fatty acids. Third, some of the 3-phosphoglycerate generated from glycolysis contributes to synthesis of the amino acid glycine, another important metabolite used to synthesize purines.

At the end of the glycolytic pathway is the three-carbon molecule pyruvate. Pyruvate has three major fates that are quantitatively important during proliferation of cancer cells (Figure 14-4). It can be reduced to lactate, transaminated to alanine, or imported into the mitochondria to be metabolized further. In proliferating cells, most pyruvate is converted to lactate, which is then secreted into the extracellular space. Some of the pyruvate is also imported into the mitochondria and oxidized further in the tricarboxylic acid (TCA) cycle. This provides more ATP for the cell, but more important, reactions in the TCA cycle allow carbon from pyruvate to be converted into other intermediates that are needed for macromolecular synthesis.

Why Is the Rate of Glycolysis So High?

In anaerobic conditions, one molecule of glucose is converted to two molecules of lactate. The terminal enzyme of anaerobic glycolysis, lactate dehydrogenase (LDH), recycles NADH back to NAD⁺, thereby allowing glycolysis to continue as long as glucose is available. But in aerobic conditions, cells need not synthesize lactate, because NADH can be recycled to NAD⁺ using shuttles that are more efficient for ATP production. Yet cancer cells display a strong preference for making lactate even when oxygen is present; Warburg himself calculated that ascites tumor cells in mice turn 30% of their dry weight’s worth of glucose into lactate in just

1 hour (8), a flux so high that he hypothesized that cancer cells must have an impairment in mitochondrial metabolism that prevented oxidative metabolism of pyruvate (9). It is now known that this is not the case for most tumors, and several other hypotheses have been proposed to explain the “aerobic” lactate production in tumors. A few of these are listed in the following paragraphs:

Capacity for High Glycolytic Flux Primes Cells to Survive Periods of Hypoxia

Rapid tumor growth may temporarily outstrip the supply of oxygen by the vasculature, requiring the nascent tumor to maintain bioenergetics anaerobically to survive. At such times, the high rate of lactate production is primarily a mechanism for survival rather than growth. Nevertheless, during subsequent stages of tumorigenesis, even after neovascularization, cells continue to exhibit high rates of lactate production. This might reflect inequality of oxygen supply among cells within the tumor, or periods of reduced oxygen delivery, demanding that some cells maintain anaerobic metabolism during those periods, and ultimately resulting in an apparently relentless production of lactate by the tumor (10,11). Cells removed from a tumor and grown in culture, where oxygen is not limiting, still produce lactate at elevated rates. This implies that lactate production is a fundamental property of the physiology of these tumors, reflecting a genetically defined alteration in metabolism.

High Rate of Glucose Consumption Is Needed to Maintain and Regulate Biosynthetic Pathways

Glucose degradation provides cells with intermediates used in a variety of biosynthetic pathways (Table 14-1). It has been proposed that tumor cells maintain robust glycolysis merely to keep pools of these intermediates high enough to support growth and that lactate production is a byproduct of the high glycolytic rate. Tumors have other potential sources of these intermediates in addition to the de novo synthetic pathways that use carbon from glucose; nonessential amino acids, fatty acids, cholesterol, and so forth are also present in the extracellular milieu. Nevertheless,

Table 14-1 Some Fates of Glucose Carbon in Growing Cells

Lactate
Nonessential amino acids
Transamination of pyruvate to alanine
Entry of glucose-derived acetyl–co-enzyme A (CoA) into the tricarboxylic acid cycle, yielding glutamate, glutamine, aspartate, etc
Transamination of glycolytic three-carbon intermediates to serine, glycine
Others
Glycerol
Three-carbon intermediates from glycolysis can be used to generate glycerol moieties of phospholipids
Fatty acids, cholesterol and other sterols, isoprenoids
Glucose-derived carbon is the major contributor to the cytosolic acetyl-CoA pool used to synthesize all these substances needed for membrane function
Nucleotides
Ribose 5–phosphate from the pentose phosphate pathway is used in synthesis of purines and pyrimidines
Some glucose carbon also contributes to the one-carbon pool used in nucleotide synthesis

tumor cells usually increase their synthetic capacity because import mechanisms cannot meet demand or de novo synthesis provides some other benefit that is not understood.

A related explanation has to do with fine-tuning the control of branched metabolic pathways. Theoretical flux analysis suggests that when a pathway branches into two downstream pathways, one with high flux and another with low flux, then the ability to regulate the latter is maximal when flux through the former is highest (12). In tumor metabolism, this has been proposed as a way to unify the apparent paradox between the need for glucose-derived carbon for biosynthesis and the apparently wasteful high flux into lactate production. In this scenario, the low-flux pathway could be any of the several points where glycolytic intermediates are withdrawn for biosynthesis. The very high rate of flux through glycolysis allows control of those other pathways to be maintained.

Lactate Secretion Creates a Microenvironment Favorable for Tumor Growth

Others have argued that cells maintain a high aerobic glycolytic rate because the lactate itself imparts a selective advantage to the tumor (13). In this model, glycolysis not only supports growth of the tumor, but also enhances its invasive potential by acidifying the environment, killing surrounding normal cells, and promoting degradation of the extracellular matrix, which normally serves to restrict invasion (14). This hypothesis predicts that suppressing LDH activity would impair tumorigenesis, and indeed this has been demonstrated in a series of experiments (15).

The Tricarboxylic Acid Cycle

The core of cellular metabolism is found in the TCA cycle (also known as the Krebs or citric acid cycle) in the mitochondria (Figure 14-5).

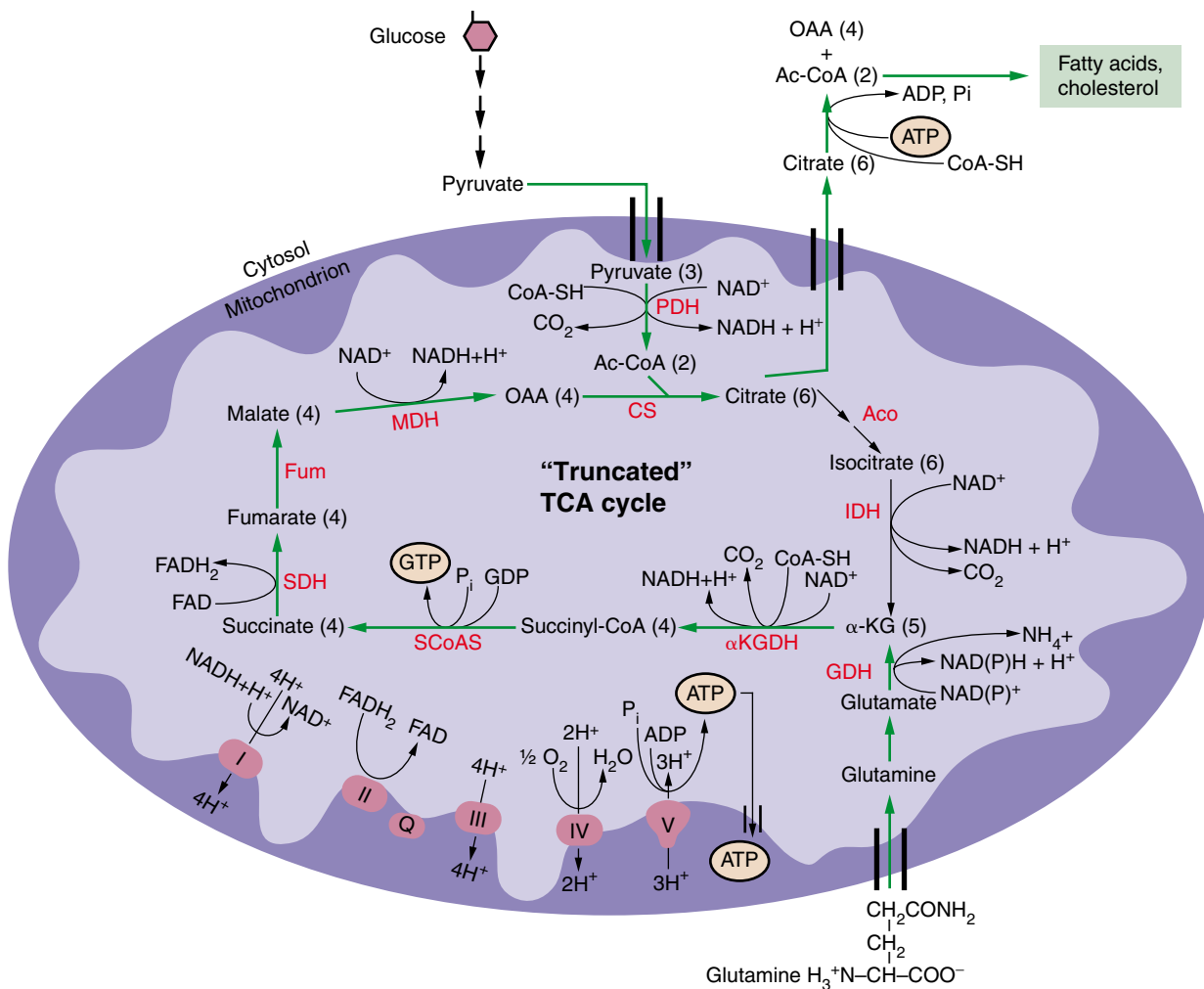


FIGURE 14-5 The tricarboxylic acid (TCA) cycle supports biosynthesis during cell growth. Pyruvate from glycolysis can enter the mitochondria, be converted into acetyl-CoA and then enter the TCA cycle by condensing with oxaloacetic acid (OAA) to form citrate, a six-carbon molecule. In highly oxidative tissues like the brain and heart, citrate proceeds around the TCA cycle, losing two carbons as carbon dioxide and eventually regenerating OAA, which can then be metabolized through further cycling. This maximizes production of adenosine triphosphate (ATP) from acetyl-CoA, as reduced electron carriers (NADH, FADH₂) contribute electrons to the respiratory chain at complexes I and II. In proliferating cells, citrate is also exported from the mitochondria to be used in the synthesis of fatty acids and cholesterol. As a result, a mechanism is required to generate OAA for further citrate production. In some cells, oxidation of glutamine fills this role. Glutamine is deaminated to glutamate in the mitochondria, and glutamate is converted to α -ketoglutarate by glutamate dehydrogenase (GDH) or other enzymes. In cells with a high rate of citrate efflux and a high rate of glutamine oxidation, the green arrows represent the predominant metabolic flux. Metabolic intermediates are in black, number of carbons in parentheses, and enzymes bolded in red. α -KG, α -ketoglutarate; α -KG DH, α -ketoglutarate dehydrogenase; Ac-CoA, acetyl-CoA; Aco, aconitase; CS, citrate synthase; CoA-SH, co-enzyme A; Fum, fumarate; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; OAA, oxaloacetic acid; PDH, pyruvate dehydrogenase; Q, ubiquinone; SCoAS, succinyl-CoA synthase; SDH, succinate dehydrogenase.

The TCA cycle is used to generate energy and convert small metabolites into precursors for biosynthetic pathways. The “entry” step of the cycle is the condensation of acetyl-CoA derived from glucose and other precursors with oxaloacetic acid (OAA), generating the six-carbon molecule citrate. In highly oxidative tissues with a large demand for ATP (e.g., heart, muscle), citrate is cycled back to OAA through a series of reactions that reduce three molecules of NAD^+ and one molecule of FAD to NADH/H^+ and FADH_2 , respectively. During this process, two carbons from citrate are lost as CO_2 . The resulting OAA can then be used to generate citrate again and start another round. NADH/H^+ and FADH_2 donate electrons to the electron transport chain, in effect maximizing ATP production from acetyl-CoA.

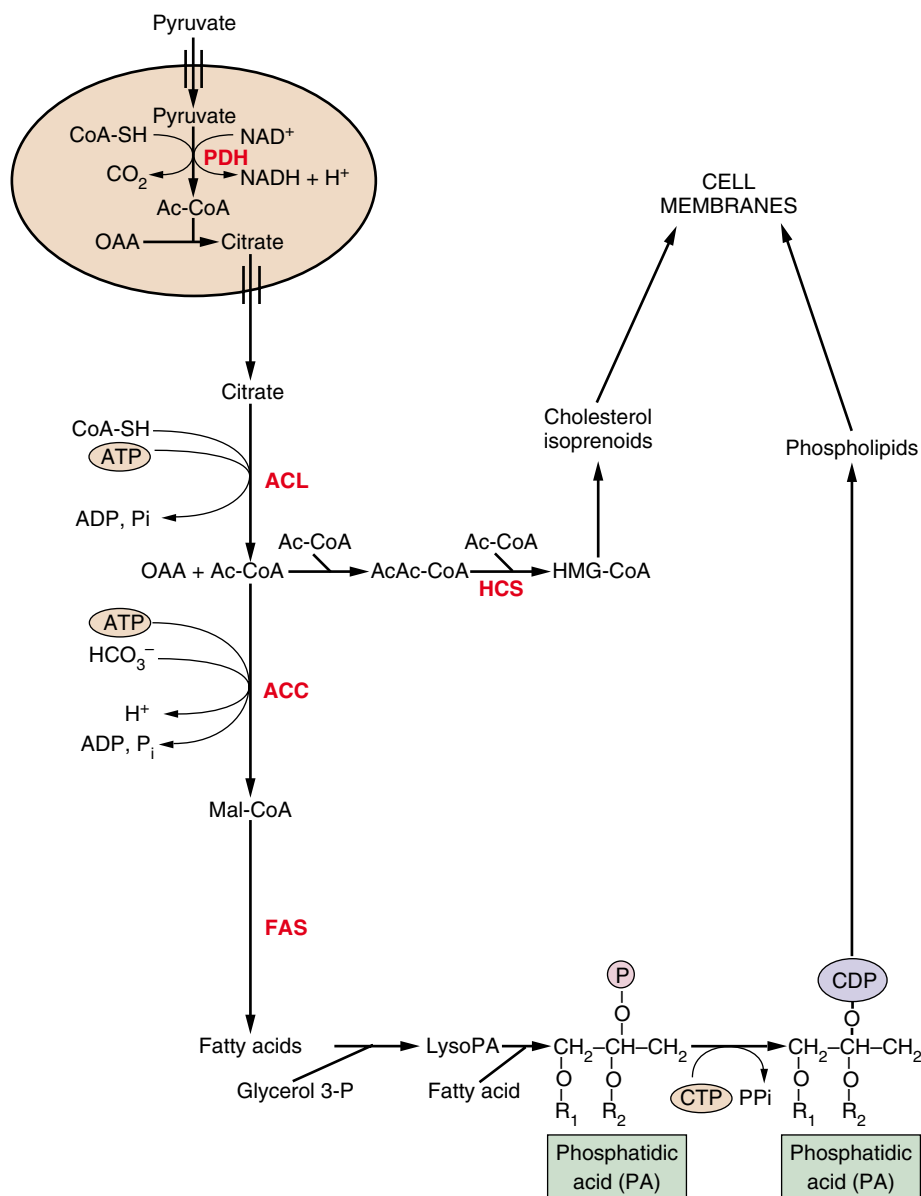
Tumors Use a “Truncated” Form of the TCA Cycle

While traditional TCA “cycling” is optimal for ATP generation, it presents a problem for proliferating cells, which need to use carbon

from acetyl-CoA and TCA cycle intermediates for biosynthetic purposes. Therefore, the carbon from a given molecule of citrate is relatively unlikely to cycle all the way back around to OAA. Instead, in tumor cells, the TCA cycle is better thought of as a biosynthetic hub, where precursors for biosynthesis are siphoned away as needed, and the cycle is refilled at downstream steps to regenerate OAA. Some investigators have referred to this phenomenon as a “truncated” TCA cycle, because the high flux of intermediates out of the pathway prevents it from acting as a true cycle.

The best-characterized examples of functional TCA cycle truncation are in the production of lipids and related molecules (cholesterol, fatty acids, and isoprenoids) needed for membrane synthesis and function. Glucose-derived acetyl-CoA is a precursor for these products, using the pathway shown in Figure 14-6, in which pyruvate from glycolysis is imported into the mitochondria, converted to acetyl-CoA, and used to form citrate. Instead of continuing around the cycle, citrate exits the mitochondria

FIGURE 14-6 SYNTHESIS OF LIPIDS AND RELATED MOLECULES USES CARBON FROM GLUCOSE. Pyruvate from glycolysis can be used to generate citrate in the mitochondria. In hepatomas and other tumors, the efflux of citrate from the mitochondria to the cytosol is an important source of acetyl-CoA for the synthesis of fatty acids, phospholipids, cholesterol and isoprenoids, all of which are required for proper membrane function. Metabolic intermediates are in black, and enzymes bolded in red. CoA-SH, co-enzyme A; Ac-CoA, acetyl-CoA; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; Mal-CoA, malonyl-CoA; Glycerol 3-P, glycerol 3-phosphate; LysoPA, lysophosphatidic acid; ACL, ATP citrate lyase; HCS, HMG-CoA synthase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; R, acyl group on lipid molecule; CTP, cytidine triphosphate; CDP, cytidine diphosphate.



and is cleaved in the cytosol, generating a pool of acetyl-CoA for synthesis of lipids. The high flux through this pathway has been carefully studied in hepatoma cells, where proliferation is proportional to the rate of mitochondrial citrate efflux and inversely proportional to citrate-stimulated respiration, a marker for traditional TCA cycling (16). Since these tumor cells are particularly rich in cholesterol, TCA truncation probably supports proliferation.

Glutamine Oxidation Makes the Truncated TCA Cycle Possible

A consequence of short-circuiting the TCA cycle to remove biosynthetic precursors is that cells now require a mechanism to regenerate OAA used for citrate synthesis. In many tumor cells, this problem is solved by oxidation of the amino acid glutamine. Tumors have long been known to consume glutamine at high rates; this is a classic observation in the field of tumor metabolism dating back to the 1950s (17). In the presence of oxygen, glutamine is oxidized in the mitochondria, entering the TCA cycle as the intermediate α -ketoglutarate. Glutamine is a major oxidizable, energy-producing substrate in tumor cells, probably exceeding even glucose as a respiratory substrate during rapid proliferation (18–21). Its participation in this pathway generates OAA in the mitochondria, allowing other TCA cycle intermediates to be used for biosynthesis.

Interruption of the TCA Cycle can be a Mechanism for Tumorigenesis

Despite abundant evidence that TCA cycling is suppressed during tumor growth, most tumor cells retain the capacity for traditional TCA activity, albeit at a rate diminished from nonproliferating cells. Because the TCA cycle is traditionally viewed as the core of cellular metabolism, it was assumed that eradication of TCA cycle activity would severely compromise cell viability. But this turns out not to be true...and surprising proof came when several types of tumors were shown to contain genetic defects in enzymes of the TCA cycle. Familial paraganglioma can be caused by mutations in *SDHB*, *SDHC*, or *SDHD*, three of the four subunits of succinate dehydrogenase (SDH), an enzyme that functions in both the TCA cycle and the electron transport chain (22–24). In affected families, a mutation in one of these genes imposes a dominantly inherited risk of paraganglioma, with loss of the wild-type allele in the tumors. This genetic mechanism is identical to that seen in classic tumor suppressors and strongly suggests that loss of SDH activity is causative rather than merely permissive for tumorigenesis in some tissues. Similarly, *SDHB* and *SDHD* mutations are among the genetic causes of pheochromocytoma (24,25). Mutations in the gene encoding the TCA enzyme fumarate hydratase (fumarase) are the major cause of a dominant cancer syndrome characterized by uterine fibroids, skin leiomyomata, and papillary renal cell cancer (26). Amazingly, in some paragangliomas caused by mutations in either *SDHB* or *SDHD*, tumor tissue contained no measurable SDH activity, proving that mutations completely abolished function of that enzyme complex, and implying that traditional TCA cycling is impossible in those cells (27,28). Despite the crippling of the TCA cycle, these cells not only survive, but accumulate at a pathologic rate. It should be emphasized that glutamine oxidation to yield OAA using the pathway in Figure 14-5 is also blocked by

these mutations. Future studies will likely clarify the mechanism of cell proliferation and the compensatory metabolism needed to maintain growth in these interesting tumors.

Nucleotide Biosynthesis: Substrate for Proliferation

To proliferate, cells perform one major biosynthetic activity that is not needed for nonproliferative growth: synthesis of nucleotides *de novo*. Proliferation requires duplication of the diploid genome, which comprises some 6×10^9 base pairs, or more than 10^{10} nucleotides. Nucleotide biosynthesis typically uses various salvage pathways in addition to the *de novo* synthesis of purines and pyrimidines. But during replicative cell division, the total number of nucleotides is doubled; therefore, *de novo* nucleotide biosynthesis is highly induced.

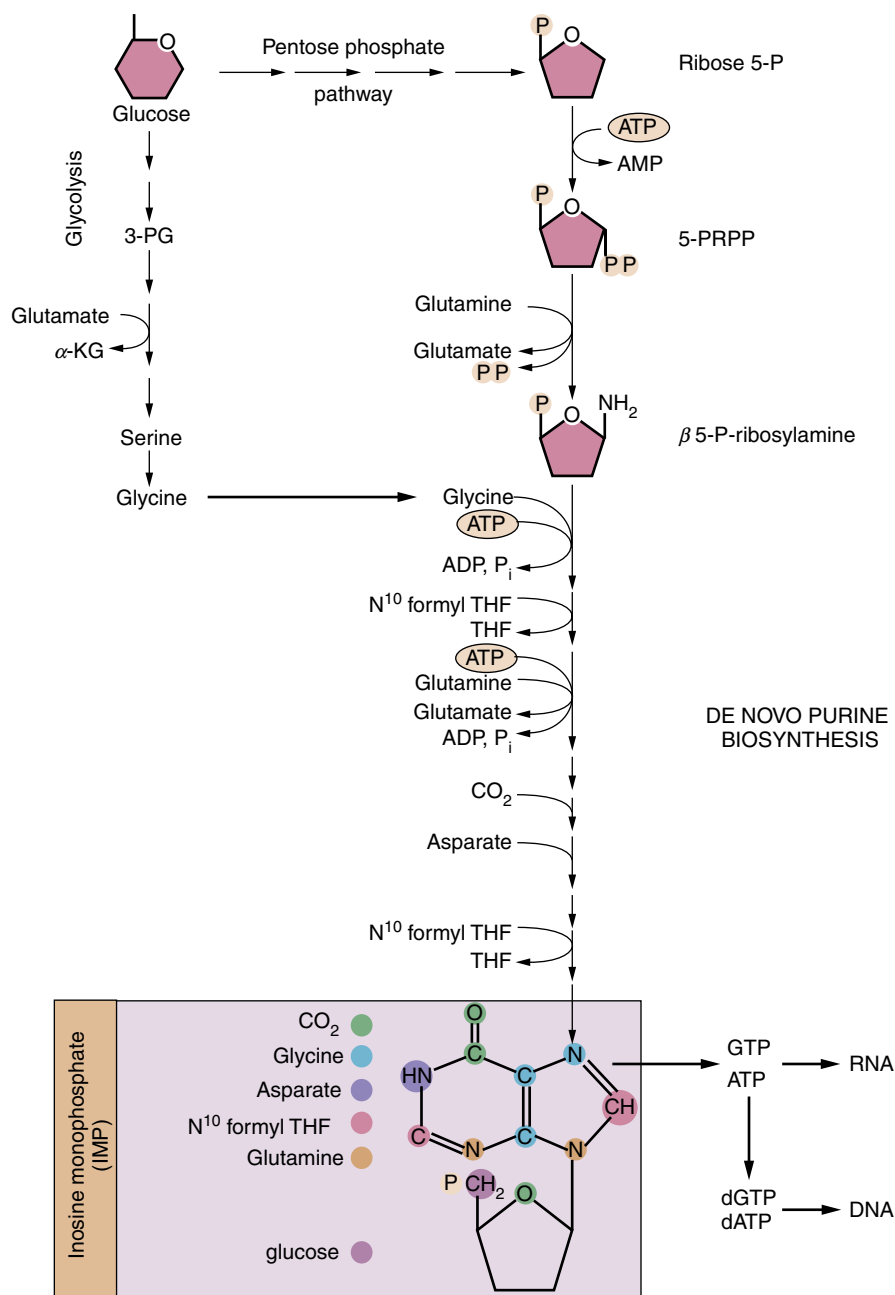
De novo biosynthesis of nucleotides is a complicated process that requires contribution of carbon and nitrogen from multiple sources, as shown in Figure 14-7 for purine synthesis (pyrimidine biosynthesis is similar, except that a free pyrimidine ring is first synthesized, then added to a ribose moiety, as opposed to the construction of the purine rings on the ribose sugar). Many of the pathways discussed above cooperate to support *de novo* nucleotide synthesis. Glucose metabolism is particularly important, because intermediates from both glycolysis and the pentose phosphate pathway are used. The sugar backbones for all nucleotides are derived from ribose 5-phosphate in the pentose phosphate pathway. Flux through that pathway also generates NADPH to produce the two N^{10} -formyl tetrahydrofolate molecules that donate formyl groups to growing purine rings. Aspartate generated from OAA in the mitochondria is used as a nitrogen donor. Two other nitrogens in the purine ring are derived from glutamine; nucleotide synthesis therefore accounts for some of the glutamine utilization observed in tumors. Finally, conversion of the resulting nucleotide into a deoxynucleotide triphosphate (dNTP) for DNA synthesis requires input of additional ATP, much of which is generated from glycolysis. Therefore, genome duplication is a major metabolic task for tumors, requiring a substantial investment of the total carbon, nitrogen and energy available to a cell.

The importance of *de novo* nucleotide biosynthesis in cell proliferation is underscored by the use of chemotherapeutic agents that interfere with this activity. In fact, this strategy is one of the oldest and most successful approaches used to treat cancer. Azaserine and 6-mercaptopurine both block the transfer of amine groups from glutamine to β -5-phosphoribosylamine, interfering with initiation of purine synthesis. Methotrexate inhibits dihydrofolate reductase, which is required to synthesize N^{10} -formyl tetrahydrofolate.

Genetic Mechanisms Behind the Metabolic Transformation in Tumors

The metabolic transformation is common to many different types of tumors arising in many different tissues, and therefore appears to be a common characteristic in tumorigenesis, promoted by the

FIGURE 14-7 NUCLEOTIDE BIOSYNTHESIS DRAWS ON SUBSTRATES FROM MULTIPLE METABOLIC PATHWAYS. Nucleotide biosynthesis is an example of why cells need to regulate multiple metabolic pathways simultaneously during cell proliferation. The de novo synthesis of purines (shown) and pyrimidines require the consumption of glucose, several amino acids, and one-carbon groups from folate metabolism. The origin of individual carbons and nitrogens on inosine monophosphate (IMP), the precursor to GTP and ATP, are color coded in the gray box. 3-PG, 3-phosphoglycerate; α -KG, α -ketoglutarate; β 5-P-ribosylamine, β 5-phosphate-ribosylamine; N^{10} formyl THF, N^{10} formyl tetrahydrofolate; PRPP, 5-phosphoribosyl pyrophosphate; Ribose 5-P, ribose 5-phosphate.



pleiotropic causes of cancer. This section discusses a few of the genetic mechanisms of human cancer and how such mutations may promote the metabolic transformation of tumors.

Activation of PI3K Pathways Clamp Cellular Metabolism in the “On” Position

The phosphatidylinositol-3-kinase (PI3K) signaling pathway is a highly conserved, widely expressed system through which cells respond to a variety of extracellular cues like lineage-specific growth factors, whose progrowth and prosurvival effects are due to their ability to signal through PI3K. Growth factor receptors that signal to the PI3K pathway and are known to be involved in tumorigenesis include Her2/Neu (breast),

platelet-derived growth factor receptor (small cell lung, prostate, etc.), endothelial growth factor receptor (head and neck, ovarian, cervical, bladder, and esophageal cancers) and others. As shown in Figure 14-8, in normal cells, binding of a growth factor to its surface receptor brings about activation of PI3K activity, resulting in phosphorylation of phosphatidylinositol (PI) species in the membrane. These are involved in recruitment and/or activation of downstream PI3K effectors, particularly the serine/threonine kinase Akt and its effector mTOR (mammalian target of rapamycin). Activation of these two molecules dramatically enhances many of the metabolic activities needed for cell growth, including import of extracellular nutrients and their utilization in pathways supporting biosynthesis of proteins and lipids (29,30).

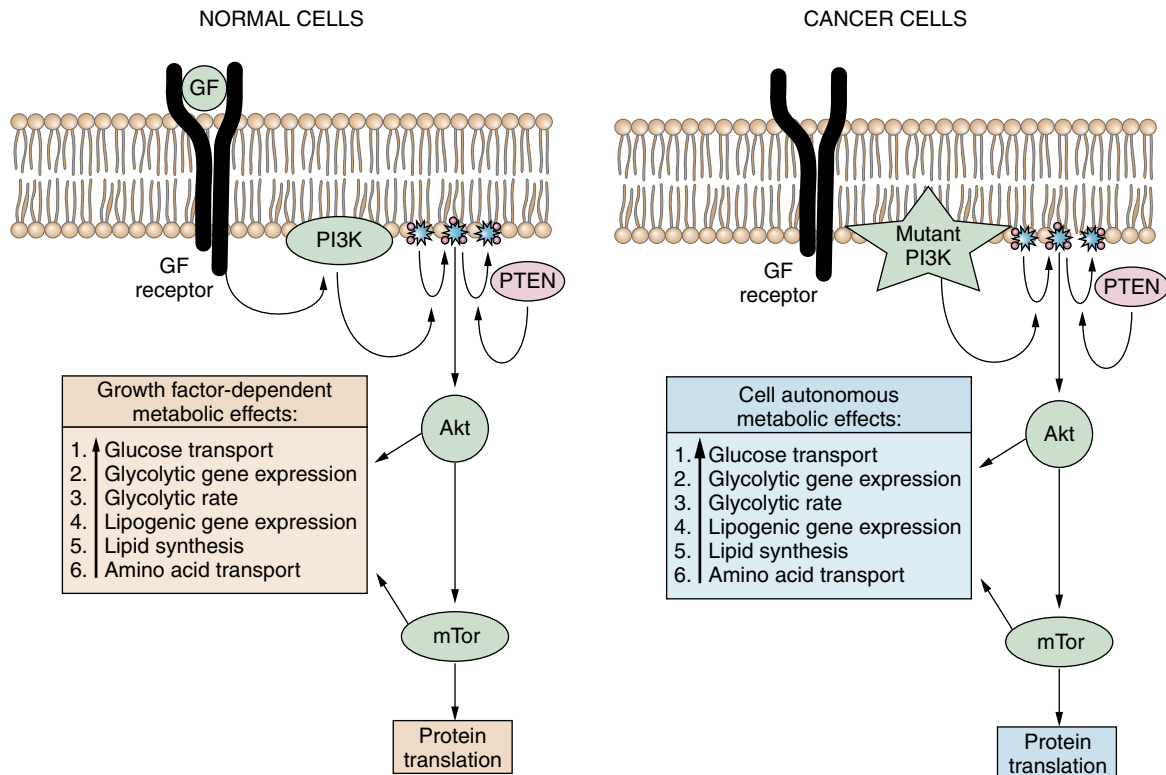


FIGURE 14-8 THE PHOSPHATIDYLINOSITOL-3-KINASE (PI3K) PATHWAY DRIVES GROWTH METABOLISM IN NORMAL AND TUMOR CELLS. In normal cells (**left**), the binding of a growth factor (GF) to its receptor initiates a signal transduction pathway that includes activation of the PI3K complex. PI3K phosphorylates membrane PI, creating lipid species like PIP_3 , which functions in the recruitment and activation of Akt. PTEN, a lipid phosphatase, dephosphorylates phosphatidylinositol (PI) species and is a negative regulator of PI3K. Activation of the serine/threonine kinases Akt and its downstream effector mammalian target of rapamycin (mTOR) elicit various metabolic effects that positively influence cell growth. Normally, in absence of the growth factor, the PI3K pathway is inactive and the cell resumes a metabolically quiescent phenotype. However, tumor cells (**right**) often contain mutations that lead to constitutive activity of the PI3K pathway, such as activating mutations in subunits of the PI3K complex itself or deletion of PTEN. These mutations enable the cell to engage in constitutive anabolic metabolism, and are a common mechanism by which tumor cells gain autonomy from growth factors.

In normal cells, activity of the PI3K system is tightly controlled by various regulatory mechanisms that titrate metabolism to needs dictated by extracellular signals. Important levels of control include feedback inhibition and dephosphorylation of PI species by the lipid phosphatase PTEN. But in many different types of malignancy, mutations bring about a chronic, stimulus-independent activation of the PI3K signaling pathway, locking cells into a metabolic phenotype of nutrient acquisition and utilization. This constitutes one of the most prevalent classes of tumorigenic

mutations ([Table 14-2](#)) and is undoubtedly an important component of the metabolic transformation. These mutations fall into three general classes: Those that directly activate PI3K subunits and their effectors; those that eliminate activity of negative regulators of the system (e.g., PTEN); and those that increase activation by introducing new or enhanced kinase activities (BCR-ABL fusion, *Her2/neu* amplification, etc.).

Regardless of the mutation, activation of Akt is likely the most important signaling event in terms of cellular metabolism,

Table 14-2 Selected Tumorigenic Mutations That Activate PI3K or Effectors

Gene	Mutation	Cancer	Frequency, %	References
<i>PIK3CA</i>	Activating point mutations	Breast	25	65
	Amplification	Colon	>30	66
		Head and neck	>35	67
<i>Akt2</i>	Amplification	Ovary	12	68
		Head and neck	30	67
<i>PTEN</i>	Mutation, loss of heterozygosity	Glioma	Up to 40	69, 70
<i>BCR-ABL</i>	Novel fusion kinase	Chronic myelogenous leukemia	>90	71
		Acute lymphocytic leukemia	20	71
<i>HER2/neu</i>	Amplification	Breast	25	72

PI3K, phosphatidylinositol-3-kinase.

because constitutive Akt activation is sufficient to drive glucose uptake, glycolysis, and lactate production in cancer cells (31), all components of the Warburg effect. How does Akt do this? It stimulates both glucose import and phosphorylation, two flux-generating steps in tumor glycolysis (32) by rapidly relocating glucose transporters to the cell surface to facilitate glucose capture and by increasing expression of hexokinase. Other steps of glycolysis are also positively regulated, resulting in a large increase in glycolytic flux. Akt also stimulates growth by inducing several biosynthetic pathways, including lipogenesis and the mTOR-mediated increase in protein synthesis.

The Transcription Factor HIF-1 Contributes to the High Rate of Glycolysis in Tumors

Tumors experience limitations in oxygen supply, and as a result, frequently exhibit zones of hypoxia that can be detected by measurements of oxygen tension (33). This phenomenon is relevant to cancer metabolism, because hypoxia is sufficient to drive some of the metabolic changes that characterize tumors, particularly the robust rates of glucose uptake, glycolysis, and lactate production. These effects are executed through HIF-1, a transcription factor complex that is stabilized and active during hypoxia. HIF-1 serves two major functions in hypoxic tumors. First, it induces the expression of vascular endothelial growth factor (VEGF), which promotes angiogenesis to reestablish tumor oxygenation. Second, HIF-1 increases expression of glucose transporters and glycolytic enzymes, accounting for many if not all of the metabolic changes induced by hypoxia.

HIF-1's involvement in cancer was established by the observation that its negative regulator, the ubiquitin ligase VHL, is mutated in von Hippel-Lindau syndrome (VHLS). The tumors of VHLS (renal cell carcinoma, paraganglioma, pheochromocytoma, hemangioblastoma) exhibit constitutive HIF-1 activity and chronic increases in the expression of HIF-1 targets, including glycolytic genes. Increased expression and/or stabilization of HIF-1 is also observed in solid tumors and metastases outside of VHL syndrome, including colon, breast, ovarian, pancreas, prostate, and others (34). In such tumors, HIF-1 expression occurs as a result of heterogeneity in oxygenation, oncogene effects, or activation of signal transduction pathways, including the PI3K pathway (35).

HIF-1's role in promoting glycolysis is clear, but it likely does not promote biosynthesis or cell growth at the cellular level. First, hypoxia suppresses protein translation through complex negative effects on mTOR, making it less likely for HIF-1 stabilization and global protein synthesis to occur concomitantly (36,37). Second, although HIF-1 activates expression of glycolytic genes, the genes needed to use glycolytic intermediates for biosynthesis do not appear to be induced (35). Third, HIF-1 curtails carbon entry into the TCA cycle by indirectly suppressing pyruvate dehydrogenase (38,39), the enzyme that converts pyruvate from glycolysis into acetyl-CoA (Figure 14-5). This increases the fraction of pyruvate converted to lactate and diminishes the ability to use pyruvate for lipid synthesis and related activities.

Therefore, the importance of HIF-1 in the metabolic transformation is complex. During tumor hypoxia, HIF-1 facilitates metabolic adaptations, allowing cells to survive and build a new vascular supply. But during growth of cells in adequate oxygenation, HIF-1 signaling is likely to suppress maximal biosynthetic activity.

Mutation of the Tumor Suppressor *LKB1* Interferes with Normal Mechanisms to Limit Growth and Proliferation

In addition to signal transduction pathways that stimulate growth, normal cells also have pathways to respond to conditions unfavorable for growth. These homeostatic mechanisms allow cells to survive periods of nutrient or energy deprivation by channeling metabolites toward energy-generating degradative pathways and away from biosynthetic pathways, which consume energy. One of the key regulators of this transition is the AMP-activated protein kinase (AMPK), which is stimulated by the high AMP/ATP ratio that occurs during energy limitation. AMPK's diverse signaling effects function to suppress growth, stimulate fatty acid and protein degradation, and induce p53-dependent cell cycle arrest (Figure 14-9), all of which allow cells to pause until their bioenergetic status is more favorable for growth (40,41).

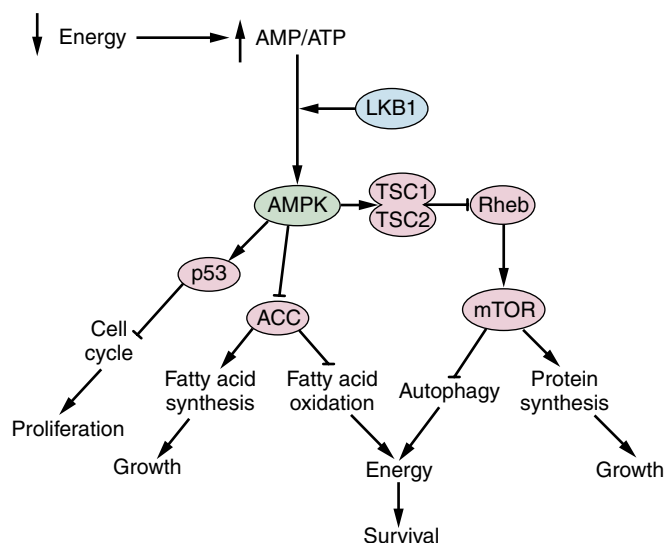


FIGURE 14-9 THE LKB1/AMPK PATHWAY ALLOWS CELLS TO RESPOND TO METABOLIC STRESS BY ENGAGING CATABOLIC METABOLISM. A decline in cellular energy status, caused by nutrient deprivation or other stressors, may result in an increase in the adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio. This activates the kinase LKB1 and its target the AMP-activated protein kinase (AMPK), a serine/threonine kinase. AMPK has numerous effects that suppress anabolic metabolism, cell growth, and proliferation, while engaging multiple catabolic pathways to restore a favorable bioenergetic state. These include phosphorylation and activation of p53, inducing cell cycle arrest; phosphorylation and inactivation of acetyl-CoA carboxylase, which suppresses fatty acid synthesis and induces fatty acid β -oxidation; and an indirect inactivation of mammalian target of rapamycin (mTOR), which serves to decrease protein synthesis and induce autophagy. Suppression of growth and proliferation preserve existing energy stores, while β -oxidation and autophagy are catabolic pathways that generate ATP.

Peutz-Jeghers syndrome (PJS) is a dominantly inherited syndrome of hamartomas, intestinal polyposis and adenocarcinoma caused by inactivating mutations of the tumor suppressor gene *LKB1*, whose product is a natural activator of AMPK (40,42). The relationship between loss of AMPK control and tumorigenesis in PJS is unclear, because *LKB1* has multiple substrates in addition to AMPK, and it participates in diverse cell process in addition to metabolism, including apoptosis, cell polarity, and cell cycle control (43). However, it is possible that *LKB1* mutation prevents cells from responding normally to environmental cues that should curtail growth and proliferation. Presumably over time, this would favor the cell accumulation that leads to formation of polyps and hamartomas.

Myc: Master Regulator of Cell Cycle Entry and Proliferative Metabolism

The Myc proteins (c-Myc, L-Myc, S-Myc, and N-Myc) are a family of transcription factors that regulate growth and cell cycle entry by their ability to induce expression of genes required for these processes (44). In normal cells, mitogen stimulation leads to a burst of Myc expression in G₁ phase, facilitating entry into the cell cycle. In transgenic mice, c-Myc overexpression increases the rate of tumor formation (45,46), and human tumors frequently exhibit increased Myc abundance relative to normal tissue. When Myc forms a heterodimer with its binding partner Max, it activates expression of a large cohort of genes, many of which have primary functions in intermediate metabolism. These include genes for the glycolytic enzymes PFK, enolase, and LDHA (47,48).

c-Myc stimulates proliferation in part by activating expression of several of the cyclins and CDK4, which promote entry into S phase (44). It is important in this regard that c-Myc's target genes also include enzymes involved in nucleotide and 1-carbon metabolism, without which cells could not successfully complete S phase. These genes include inosine 5'-monophosphate dehydrogenase (49), serine hydroxymethyl-transferase (50), adenosine kinase, adenylate kinase-2, and phosphoribosyl pyrophosphate amidotransferase (51). Therefore, c-Myc may perform dual functions in S-phase control: It coordinates the signaling that governs the G₁/S transition and enhances the metabolic activity needed to execute genome replication.

Clinical Aspects of Tumor Metabolism

Ultimately, the goal of research in tumor metabolism is to exploit differences between tumors and normal tissue for diagnostic and therapeutic benefit. The future holds promise for significant advances in this regard, as convergence of information from gene expression and metabolic profiling of tumor tissue may reveal attractive metabolic targets for drug development. But even at present, tumor metabolism, specifically the prominent role of glucose metabolism, plays a key role in treating cancer patients.

PET is a nuclear medicine imaging modality that allows metabolism to be interrogated in vivo with the use of radioactive tracers. The most commonly used tracer in cancer is [¹⁸F]fluorodeoxyglucose (FDG), a glucose analog that can be transported into cells and phosphorylated by hexokinase, but cannot be metabolized further. Therefore, cellular import and phosphorylation of FDG traps it inside cells, and whole-body scanning then allows the observer to distinguish regions of abnormally high glucose metabolism. Because of the robust glucose metabolism during cell proliferation, tumors act as FDG sinks, making PET useful for a variety of cancers, including breast, colorectal, lung, brain, ovarian, and lymphoma (52). FDG-PET is now one of the most versatile clinical tools in the management of certain cancers. It can be used to identify new tumors, to determine regional lymph node involvement or diagnose distant metastases, and to evaluate response to therapy. The latter application is particularly promising because an attenuation of FDG signal, a positive indicator of tumor response, has been observed within a few days after initiating therapy for some tumors (53,54). By contrast, a reduction in tumor size by conventional imaging techniques (computed tomography [CT], magnetic resonance imaging [MRI]) usually takes weeks to months. Future research on the use of FDG-PET and other metabolic imaging modalities should broaden their use in cancer patients.

The dependence of some tumors on glycolysis also appears to be one of the mechanisms that render such cells susceptible to alkylators and other DNA-damaging agents. Normally, a moderate amount of DNA damage brings about activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which adds ADP-ribose polymers to its multiple target proteins (55). This facilitates repair of the damaged DNA through mechanisms that are not completely understood. However, PARP must obtain ADP-ribose subunits by hydrolyzing NAD⁺. If DNA damage is extensive, PARP can deplete the cytosolic NAD⁺ supply, and this impairs glycolysis in vitro (56,57). Therefore, cells with the highest dependence on glycolysis for energy and other processes do not survive extensive DNA damage. This has been proposed to be one of the major sources of cell death in this context: the reliance of proliferating tumor cells on glycolytic metabolism acts as an "Achilles heel" when they are exposed to agents that extensively damage DNA (58).

Ongoing efforts are dedicated to determining whether tumors have other metabolic vulnerabilities that can be exploited clinically. One example that has been successfully used in animal models of cancer involves the truncated TCA cycle and the reliance of tumors on de novo fatty acid synthesis. These studies were motivated by the observation that tumor tissue expressed high levels of the enzymes required for this particular pathway (59–62). When these enzymes were inhibited using drugs or genetic manipulations, tumor cell proliferation was suppressed in vitro. More importantly, inhibition of these enzymes also slowed the growth of tumors in vivo (63,64). Approaches like these, inspired by careful study of the metabolic activities of tumors, hold out the most promise for rational design of metabolically directed therapies that will be effective and well tolerated.

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Apoptosis, Autophagy, and Necrosis

Cell death is one of the fastest growing fields in cancer research. It is now well recognized that a fundamental characteristic of multicellular organisms is that some cells must die for proper development to occur and to maintain homeostasis and health. This propensity to die for the good of the organism has evolved so that cells are systematically dismantled through a hard-wired response termed “programmed cell death” (PCD). The number of cells in an organism is tightly controlled by an exquisite balance between proper cell proliferation, differentiation, and cell death. Indeed, in mammals billions of epithelial and blood cells die every day. On the surface, the enormity of cell death in multicellular organisms seems incredibly wasteful, yet these processes play essential roles in maintaining the homeostasis that ensure that individual tissues maintain their correct size and proper function.

All eukaryotic cells can undergo the cell death response, which can be triggered by internal or external stimuli. Important examples of this phenomenon are seen in vertebrate development during the sculpting of fingers where the cells between digits are cleared through cell death and in the selective removal of autoreactive lymphocytes. Similarly, cell death plays an important role in regulating blood cell numbers. Blood cell progenitors are continuously made in excess in the bone marrow, yet these progenitors, and their progeny, are cleared by cell death, which prevents overproduction and disease states such as leukemia, lymphoma, and/or lympho-, myelo- or erythrocytosis. In the case of erythrocytes, this excess again seems incredibly inefficient, yet plays an important role in keeping the organism prepared for times of hypoxia induced by rapid blood loss due to injury or following exposure to agents that provoke anemia. Here erythrocyte progenitors can be quickly rescued from the cell death program by increases in the hormone erythropoietin, which inhibits cell death and promotes the differentiation of these progenitors into erythrocytes. These examples underscore the importance of balancing cell proliferation, differentiation, and cell death. Indeed, when this balance goes awry, disease ensues.

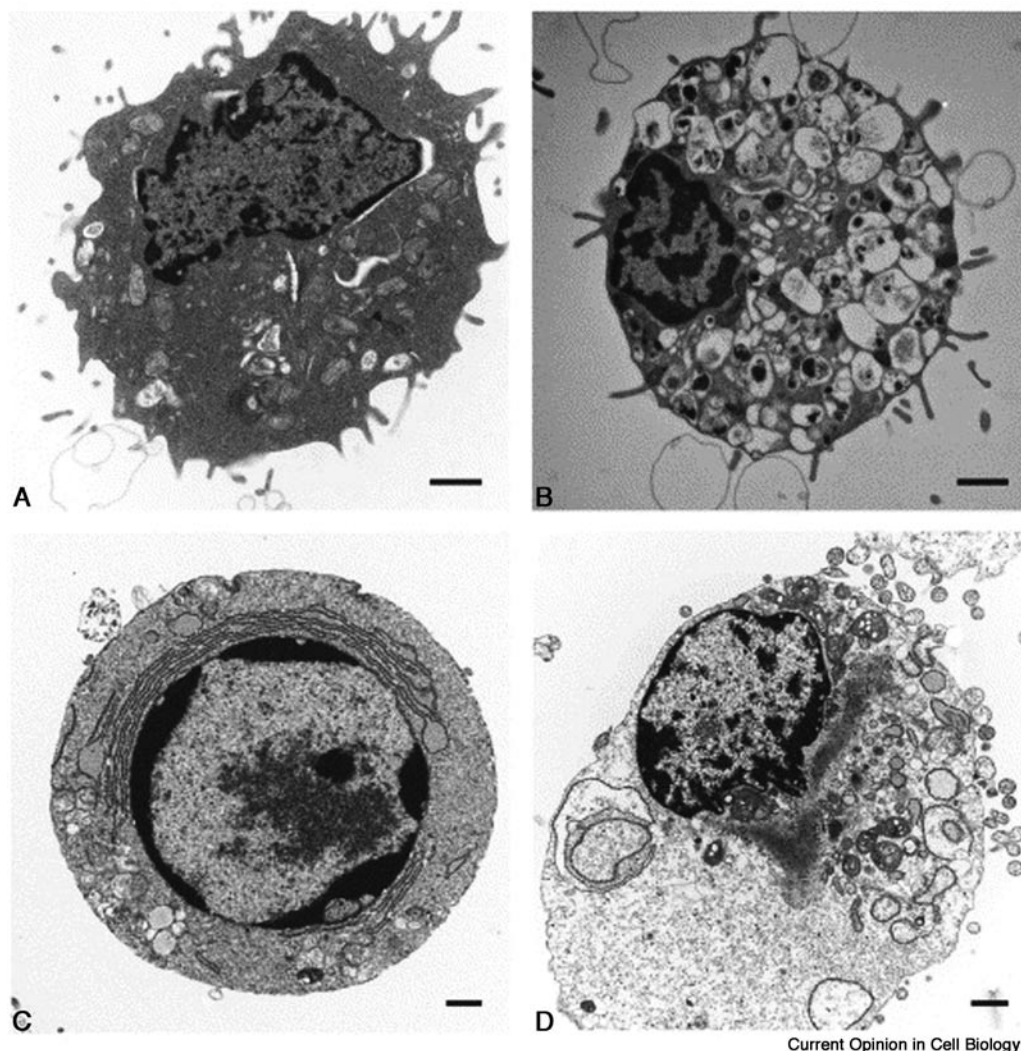
A defining characteristic of a cancer cell is its ability to resist cell death. The resistance of tumor cells to death is not complete, but rather confers an enhanced ability to survive under conditions of cell stress. This comes into play in the tumor microenvironment, often hypoxic or nutrient poor or when such cancer cells are faced with chemotherapeutic agents or exposed to irradiation. In general, the major hurdle in treating cancer is the inability to

selectively kill cancer cells over normal, healthy tissue. Acquired resistance to cell death is a hallmark of late-stage, metastatic malignancies. In fact, most of the side effects of traditional chemotherapy results from the induction of programmed cell death in normally dividing tissues, such as the intestinal epithelium and bone marrow. Understanding the molecular mechanisms that induce cell death is thus essential for the development of new chemotherapeutic regimens that are effective in cancer treatment and prevention.

Although programmed cell death was recognized over a century ago, the signaling pathways and molecular mechanisms that govern the demise of the cell have only recently been unmasked. In the 1970s, electron microscopic analyses of dying cells led to the classification of at least three different forms of cell death that are morphologically distinct (Figure 15-1). These cell death pathways included apoptosis, autophagy, and necrosis. Apoptosis and autophagy efficiently destroy the cell from within, whereas necrosis results in the loss of the integrity of the plasma membrane liberating intracellular contents into the extracellular milieu. All three of these pathways are now known to be highly regulated processes that play essential roles in both development and homeostasis. They all also play critical roles in pathologic states such as ischemia, neurodegeneration, acute infection, autoimmune syndromes, and cancer. In such scenarios the functions of these pathways dictate whether the organism itself lives or dies. Accordingly, mutations in the genetic pathways that control cell death have been revealed to be major contributors to disease states, particularly in the development and resistance of cancer. In this chapter, we provide an overview of these cell death pathways, their regulators, and their mechanisms of action, and specifically explore their relationships to the development and treatment of cancer.

Apoptosis

The cells of all metazoans harbor the hardware necessary to initiate and execute PCD when triggered by specific stimuli, in effect, cell suicide. The best characterized form of PCD is called apoptosis, a Greek term that is loosely translated as “falling off or away.” Apoptosis was initially used to describe the morphologic sequence of events that accompany cell death, which grossly resembled the shrinkage and withering of tree leaves in autumn. Since its



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FIGURE 15-1 THE MORPHOLOGIES OF CELL DEATH. Morphologic characteristics of a normal cell (A) compared with cells undergoing (B) autophagic, (C) apoptotic, and (D) necrotic cell death. Although the morphologic characteristics of an apoptotic cell are well defined, autophagic vesiculation can be seen in all three forms of cell death. In the context of apoptosis or necrosis, autophagy could be additive or may serve to protect cells from death. Indeed, bioenergetic failure, which will lead to necrosis, can be thwarted by the up-regulation of autophagic degradation to maintain proper adenosine triphosphate levels.

morphologic description, major headway has been made into understanding the molecular wiring of apoptotic programs. The simplest definition of apoptosis is as an energy (adenosine triphosphate [ATP])–dependent form of programmed cell death, regulated by specific genes and their encoded proteins, which results in the neat and orderly destruction of the cell from within, thus also preventing undesired inflammation.

Cells undergoing apoptosis are distinguished by a set of unique morphologic and biochemical changes. The first noticeable difference in cellular morphology is cell shrinkage. Accompanying this event, adherent cells lose their contacts with the substratum and with their neighbors, which results from the proteolytic breakdown of the cytoskeleton. The nucleus in an apoptotic cell undergoes prominent morphologic changes as well; chromatin condenses and localizes to the edge of the nuclear membrane, while the nucleolus becomes enlarged and granular. Chromatin is also cleaved by a double-stranded endonuclease that cuts the genome between its nucleosomes, resulting in ≈ 180 base pair fragments,

and multiples thereof, which look like a ladder when analyzed by electrophoresis on an agarose gel. Nuclear shrinkage (pyknosis), fragmentation (karyorrhexis), and DNA laddering are considered classical morphological signs of apoptosis.

An important feature of apoptotic cells is that their membranes remain intact, but they are partitioned into many small membrane vesicles, called apoptotic bodies, that literally bleb out from the surface of the cell and contain intracellular components. Furthermore, during apoptosis the lipid phosphatidylserine, which is normally localized almost exclusively on the inner leaflet of the cell membrane, is translocated to the outer leaflet by a “flip-pase” called aminophospholipid translocase (1). The final throes of this death response include the engulfment of apoptotic bodies by neighboring cells and macrophages, directed by receptors that specifically recognize phosphatidylserine; this ensures a clean execution and prevents the release of intracellular components into the environment, which would otherwise induce an inflammatory response.

Caspases: The Executioners

The initiation of the apoptotic program is regulated either by intrinsic signals that depend on intracellular mediators or is regulated by extrinsic signals that rely on the interactions of extracellular ligands with specific transmembrane “death” receptors. The intrinsic pathway is activated by many forms of intracellular stress, including the deprivation of nutrients, or requisite growth survival factors, oncogenic “stress” (see subsequent sections), damage to DNA or proteins caused by exposure to irradiation, reactive oxygen species, or chemotherapeutic drugs, hypoxia, and endoplasmic reticulum (ER) stress. By contrast, extrinsic pathways include those triggered by ligand binding to the Fas family of death receptors or toxic proteins such as perforin and granzyme-B released from cytotoxic lymphocytes and natural killer cells, which literally blow holes in their cellular targets.

Intrinsic and extrinsic apoptotic pathways converge on a cast of highly specific and conserved aspartate-specific, cysteine proteases termed “caspases,” which are the key effectors of the apoptotic response. Caspases are expressed as zymogens of ≈ 30 to 50kD, and generally contain an N-terminal prodomain, a large subunit, and a small subunit. These zymogens become activated either through self-cleavage events or cleavage by other, upstream caspases (2). Following cleavage, the large and small subunits then associate to form the mature enzyme, which specifically recognizes select tetrapeptide peptide sequences having a C-terminal aspartate residue. The physiologic function of a few of the caspases (e.g., caspase-1) is to cleave cytokines from their pro- to active forms, and this response plays important roles in inflammation. However, the remainder function as executioners of PCD, and include both initiator and effector caspases, which differ in their substrates. Specifically, initiator caspases cleave and activate effector caspases, which then cleave key targets required for cellular integrity (Figure 15-2; 2).

Unlike other post-translational modifications, proteolysis is irreversible, and as a consequence, full caspase activation represents “a point of no return” for the dying cell. Initiator caspases such as caspase-8, -9, and -10 are the first to be activated in response to apoptotic stimuli, and this occurs through their recruitment to scaffolding proteins, which increases their effective local concentration (3). This then provokes cross (self)-cleavage and activation. For example, in extrinsic apoptosis, death receptors such as Fas first trimerize in response to binding to (membrane-bound) Fas ligand. This clustering facilitates the binding of an adaptor molecule coined FADD (*Fas-associated death domain protein*; 4), which occurs through protein–protein interactions directed by the “death domains” of Fas and FADD. Following recruitment to the receptor, FADD then forms higher-order oligomers, which in turn recruit procaspase-8 to form the so-called death-inducing signaling complex (DISC). Procaspase-8 normally exhibits low levels of activity, yet the DISC provides a scaffold that facilitates its self-cleavage, and activated caspase-8 then cleaves its downstream substrates (Figure 15-3).

In a similar fashion, during intrinsic forms of apoptosis, procaspase-9 is activated by binding to a scaffold protein coined Apaf-1 (3), which normally resides in the cytosol. Apaf-1 is composed of an N-terminal caspase recruitment domain (CARD), a central ATP-binding domain, and C-terminal WD40 repeats (2). Normally, Apaf-1 is kept as an inactive monomer through the intramolecular interactions of its CARD domain and WD40 repeats. However, triggers of the intrinsic apoptotic pathway ultimately provoke permeabilization of the mitochondrial membrane and the subsequent release of cytochrome *c*, as well as a host of other pro-apoptotic molecules, from the inner mitochondrial membrane space. Once released, cytochrome *c* binds to Apaf-1 and, with ATP hydrolysis, Apaf-1 then heptamerizes to recruit caspase-9, forming a large wheel-like structure termed the “apoptosome”

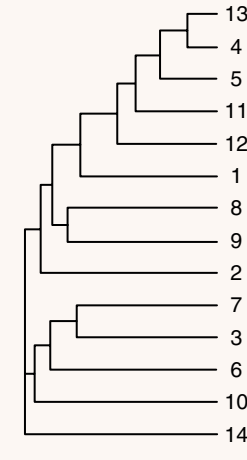
Sequence Homology	Function	Adaptor Molecule
	Inflammation? Inflammation Inflammation Inflammation Inflammation Inflammation Initiator Initiator Initiator/Effector Effector Effector Effector Initiator Effector?	TRAF-2 Ipaf, CARD-8, Nod-1 FADD, DEDAF, ASC Apaf-1, Nod-1, PACAP RAIDD, PACAP, DEFCAP FADD, DEDAF

FIGURE 15-2 CASPASES. The caspase family of proteases shows a high degree of homology and is divided into initiator, effector, and cytokine processors. Initiator caspases are the proximal proteases that regulate caspase-dependent cell death and are activated by adaptors such as FADD and Apaf1, which provide platforms on which these initiator caspases can cleave one another, resulting in their full activation. Once activated, initiator caspase then cleave the proforms of the effector caspases, which then cleave key targets required for cell integrity. Together initiator and effector caspases lead to the biochemical and morphologic changes that are hallmarks of apoptosis. Interestingly, caspases involved in inflammation cosegregate on the basis of homology, indicating an evolutionary divergence in the function of this family of proteases.

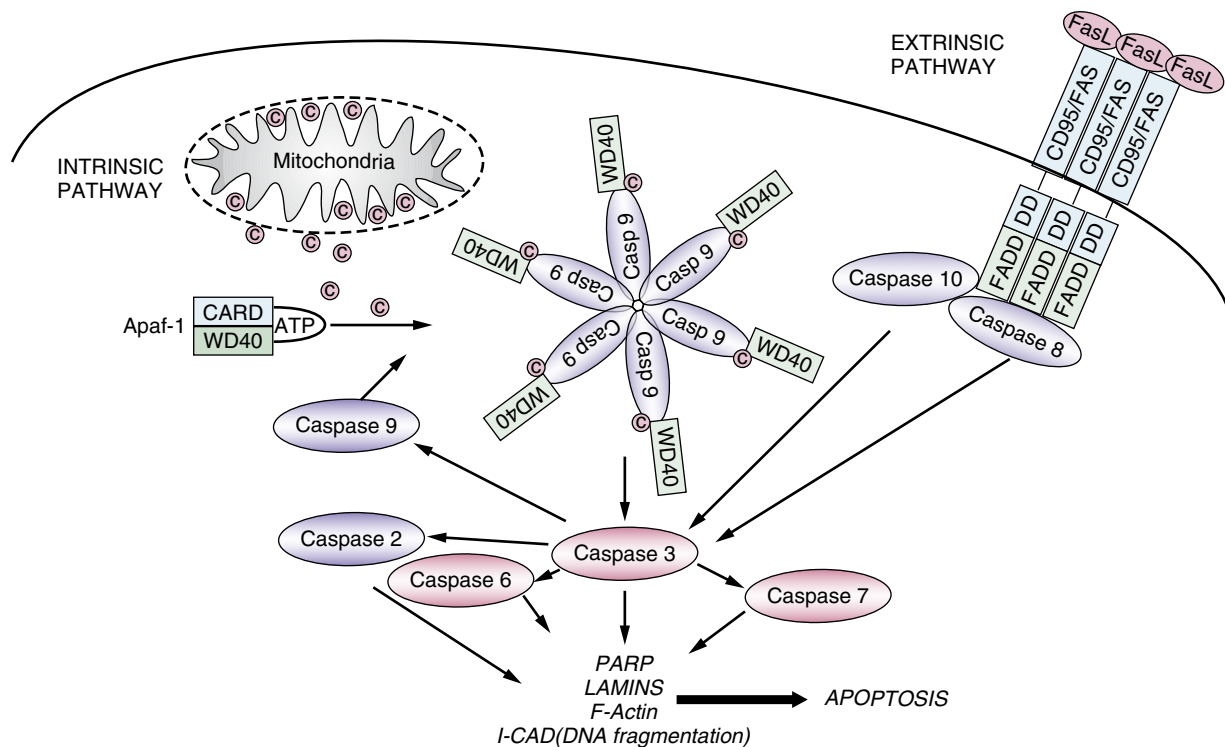


FIGURE 15-3 ACTIVATION OF CASPASES BY THE INTRINSIC AND EXTRINSIC APOPTOTIC PATHWAYS. The intrinsic apoptotic pathway is regulated by mitochondrial outer membrane permeabilization (MOMP), which results in the release of cytochrome *c* (red circles), which then binds to the WD40 domain of monomeric Apaf-1. Together with the hydrolysis of adenosine triphosphate (ATP) and another round of ATP binding, Apaf-1 then recruits the initiator caspase (purple ovals) caspase-9 to the heptameric apoptosome via its CARD domain, facilitating caspase-9 activation. Caspase-9 then cleaves the effector caspase (red ovals), caspase-3, which then cleaves key targets required for cell integrity. The extrinsic pathway is initiated by the binding of ligand (FasL) to Fas receptor, which trimerizes the receptor. This results in the recruitment of FADD, which in turn recruits and activates the initiator caspases-8 and -10, which then cleave and activate effector caspases, which then direct the destruction of the cell.

(Figure 15-3; 5). As with caspase-8, the recruitment of caspase-9 results in its self-cleavage and activation.

Once activated, caspases-8 and -9 cleave and activate effector caspases such as caspase-3, -6, and -7, which in turn cleave a wide array of proteins required for cell integrity (2). Such caspase substrates include cytoskeletal proteins such as actin and fodrin, as well as gelsolin, which when cleaved then severs actin filaments. Also targeted are structural components of the nuclear membrane such as lamin-A, lamin-B, and chromatin, via degradation of the inhibitor of caspase-activated deoxyribonuclease (ICAD), which allows CAD to direct internucleosomal cleavage of chromosomal DNA.

Caspase activation is further regulated by direct caspase inhibitors and inhibitors of those caspase inhibitors. Indeed, some direct caspase inhibitors such as the IAP (inhibitor of apoptosis) family members XIAP, cIAP-1, cIAP-2, Survivin, ILP-2, and Livin are up-regulated in select cancers, and this has been shown to render the tumor cell more resistant to apoptosis induced by chemotherapeutic agents (6). Although the precise mechanism(s) by which some of these proteins inhibit caspases is unclear, XIAP and Survivin have been clearly shown to bind to and inhibit activated caspase-3 and caspase-7 (6). Other inhibitors function at the level of initiator caspases, and most prominent amongst these is a caspase-8 inhibitor coined “FLIP”, which harbors two “death

domains” that bind to the DISC, thereby inhibiting the recruitment and activation of caspase-8. Finally, in addition to cytochrome *c*, permeabilization of the mitochondrial membrane results in the release of other factors that also target caspases. Notably these include inhibitors of IAPs such as Smac/DIABLO (which stands for “second mitochondria-derived activator of caspase/direct IAP binding protein with low pI”) and Omi/HtrA2 (high temperature requirement A2 protein; 6).

As caspases are integral components of the apoptotic machinery, they are frequently targeted during tumorigenesis. Nonsense, frame-shift, and missense mutations have been identified in *caspase-8* in invasive colorectal tumors, and somatic mutations are common in *caspase-7* and -10 in hematologic malignancies and gastric tumors, respectively (reviewed in [6]). Interestingly, although *caspase-10* mRNA is present in several pediatric tumors, the protein is not produced, suggesting that there are mechanisms that target the translation and/or turnover of some caspases. In addition, a number of the components of the caspase pathway are silenced in a large number of cancers through epigenetic means. For example, *Apaf-1* is silenced in some forms of acute and chronic myeloid leukemia, melanoma, and acute lymphoblastic leukemia, whereas *caspase-8* is silenced in several pediatric tumors, including neuroblastoma, rhabdomyosarcoma, medulloblastoma, and

retinoblastoma. Furthermore the expression of caspases-1, -2, -3, -6, -7, -8, -9, and -10 is repressed in multiple cancer lines and in neoplastic tissues when compared with normal tissue. Thus, restoration or enhancing the expression of caspases or their regulators may be therapeutic. In support of this notion, enforced expression of caspase-3 in deficient cancer cell lines increases their sensitivity to chemotherapeutic agents, and treatment of chemoresistant, metastatic melanomas with 5-aza-2'-deoxycytidine, an inhibitor of gene methylation, restores Apaf-1 expression and sensitizes these tumors to chemotherapeutic agents. Finally, some caspases also undergo alternative splicing to produce isoforms that can oligomerize with and inhibit endogenous caspases, and several truncated caspase isoforms are up-regulated in cancer.

The Bcl-2 Family of Cell Death Regulators

The gatekeepers of mitochondrial-dependent apoptosis are the Bcl-2 family of apoptotic regulators that regulate cell death and survival. The founding member of this family, *Bcl-2*, was identified as an overexpressed gene found in the t(14;18)(q32;q21) translocation (7), a hallmark of follicular B-cell lymphoma. *Bcl-2* was classified as an oncogene because its overexpression can drive or promote tumorigenesis, yet unlike all other oncogenes *Bcl-2* conferred resistance to cell death rather than driving cellular proliferation. It is now clear that enhanced cellular proliferation and resistance to cell death are both necessary for tumorigenesis and that the combination of these two classes of oncogenes has lethal consequences.

As the more than 20 Bcl-2 family members were identified, it became evident that they all share α -helical domains (BH1–4) homologous to those present in Bcl-2 (Figure 15-4). This family is subdivided into three groups on the basis of their structure and anti-apoptotic or pro-apoptotic functions (reviewed in [8]). First, the anti-apoptotic members Bcl-2, Mcl-1, Bcl-X_L, Bcl-w, and A1 have all four BH domains, and the BH4 domain is specifically required for their anti-apoptotic functions. The second group consists of Bax, Bak, and Bok, which contain the BH1–3 domains, and which function as pro-apoptotic regulators. Members of both of these groups usually have a transmembrane domain in their

C-terminus, and they regulate the release of calcium from the endoplasmic reticulum (ER) and pro-apoptotic molecules such as cytochrome *c*, Smac/DIABLO, and Omi/HtrA2 from mitochondria. Further, these molecules can homo- and hetero-oligomerize via their BH1–3 domains, and these interactions form a pocket that is binding site for other BH3-domain containing proteins. The final group consists of the BH3-only family members, which include Bid, Bim, Bad, Bik, Noxa, and Puma, among others. These proteins function as signaling entities that tip the balance toward death in response to specific intracellular stresses, which generally occur through specific binding to and engaging of anti-apoptotic Bcl-2 proteins, rather than by directly activating Bax or Bak.

Bax and Bak, via their BH3 domains, form homo- and hetero-oligomers that mediate cell death by forming pores in the mitochondrial outer membrane, which result in its permeabilization. Bax's transmembrane domain is normally buried by intramolecular interactions, and as a result, Bax is cytoplasmic under normal conditions. However, following the induction of apoptosis, Bax's transmembrane domain inserts into the mitochondrial outer membrane, and it then oligomerizes to initiate membrane permeabilization. It is now generally thought that anti-apoptotic Bcl-2 family members inhibit this process through their ability to heterodimerize with Bax and Bak and that this function is disrupted by the binding of BH3-only proteins to anti-apoptotic proteins (9). Although the precise mechanism is not completely understood and is still hotly debated, it is clear that the ratio of pro-apoptotic to anti-apoptotic members plays an essential role in the decision to die. For example, gene-targeting studies have demonstrated that cells from mice lacking both *bax* and *bak* are resistant to all forms of intrinsic and extrinsic apoptosis and, accordingly, these doubly deficient mice usually die soon after birth (10). Furthermore, Bcl-2 and other anti-apoptotic family members such as Bcl-X_L and Mcl-1, have been shown to be up-regulated either directly (e.g., *BCL2* chromosomal translocations in follicular lymphoma; 7) or indirectly in several cancers, where they confer a profound resistance to chemotherapy and radiation (11). In addition, gene knock-out studies have demonstrated that *bcl-2* is essential for the survival of mature lymphocytes (12), whereas *bcl-X* plays key roles in

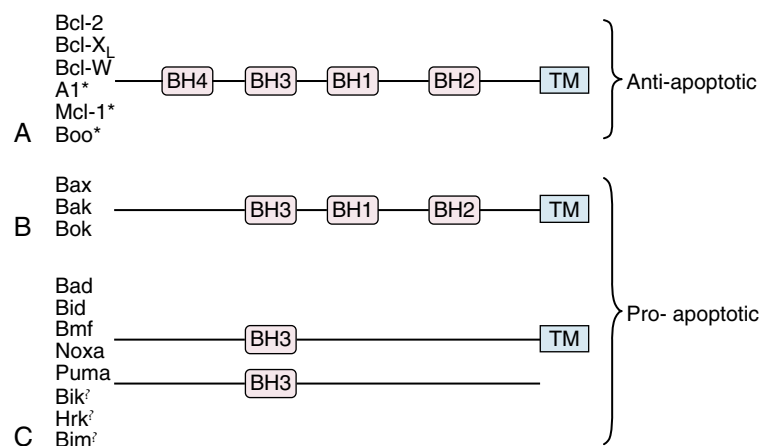


FIGURE 15-4 BCL-2 FAMILY OF APOPTOTIC REGULATORS. The Bcl-2 family is separated into three subfamilies: (A) the multidomain anti-apoptotic subfamily, most of which contain four Bcl-2 homology (BH1, BH2, BH3, and BH4) domains and includes Bcl-2 and Bcl-X_L; (B) the multidomain pro-apoptotic subfamily, which all contain three BH (BH1–BH3) domains, and includes the gatekeepers of apoptosis Bax and Bak; and (C) the pro-apoptotic BH3-only subfamily, which only harbor a single BH3 domain. Some members of each of the subfamilies contain members that have transmembrane domains (TMs), which facilitate their association with membranes, such as the outer membrane of mitochondria.

immature lymphocytes and in hematopoietic progenitors (13). Finally, deletion of *Mcl-1* leads to very early embryonic lethality and the conditional knock-out of *Mcl-1* demonstrated that it also has nonredundant, essential roles in most hematopoietic cell lineages (14). Therefore, the anti-apoptotic Bcl-2 family members also play key roles in controlling developmental cell survival.

Apoptosis induced by BH3-only proteins requires Bax and Bak, and with the exception of Bid, BH3-only proteins function by binding to and inactivating anti-apoptotic family members such as Bcl-2 (8). BH3-only proteins are held in check by multiple mechanisms, including cytosolic sequestration (Bim and Bif), phosphorylation (Bad), proteolytic cleavage (Bid), and transcriptional repression (Puma, Noxa, and Hrk; 3). Knock-out studies support the notion that BH3-only proteins act as sentinels of specific intracellular stress cues that activate the apoptotic machinery. For example, Noxa and Puma are both induced by the p53 tumor suppressor in response to genotoxic or oncogenic stress. Similarly, Bim is required to maintain proper numbers of lymphocytes (15), whereas Bid is required for Fas-induced cell death in hepatocytes. Bid is unique in that it can be cleaved to smaller forms by caspase-8 (e.g., to tBid), granzyme-B, or calpain. In turn, tBid may bind directly to Bax or Bak, and promote Bax membrane insertion and Bax/Bak oligomerization. Indeed, tBid provides a direct link between the extrinsic and intrinsic apoptotic pathways (Figure 15-4).

An obvious prediction based on their function is that pro-apoptotic Bcl-2 family members would behave as classic tumor suppressors. Indeed, *Bax* (16) and *Bak* (17) are inactivated by somatic mutations, and *bax* loss accelerates the course, and modifies the tumor spectrum, in several mouse cancer models. However, in *bax* heterozygous tumor-prone mice, the wild-type allele has not been demonstrated to be lost or silenced, indicating that Bax does not behave as a classical tumor suppressor, but rather as a modifier of malignancies (18). Nonetheless, other pro-apoptotic family members are mutated (Bim) or silenced (Noxa) in malignancies, and loss of *Bik* has been proposed as a hallmark of renal cell carcinoma (8). Thus, strategies that activate BH3-only proteins specifically in cancers may also prove to have therapeutic benefit.

Death Receptors

The extrinsic apoptotic pathway connects the cell death machinery to the extracellular milieu, and this triggers cells to commit suicide when their death receptors are bound by their cognate ligands. This pathway relies on a family of more than 20 type I transmembrane death receptor proteins, which are characterized by a cysteine-rich extracellular domain, and a short cytoplasmic (≈ 80 residue) domain that contains the death domain (DD). The best characterized members of this family include tumor necrosis factor- α TNF- α receptor 1 (TNF-R1), Fas (Apo-1/CD95), TRAIL-R1 and -R2, DR3, DR6, as well as the p75 nerve growth factor receptor, all of which contain a DD that can trigger apoptosis (reviewed in [19]). The ligands for these receptors are almost exclusively type II transmembrane proteins, yet these can be cleaved by metalloproteinases to generate soluble forms of these ligands.

Once engaged by membrane-bound ligand, death receptors then trimerize, recruit FADD, and form the DISC, which serves as a platform for recruiting and activating the initiator caspases-8 and -10 (Figure 15-5; 19).

Death receptor ligands such as TNF- α , FasL, and TRAIL are potent inducers of apoptosis, and play important roles in tissue homeostasis and tumor development and maintenance (19). For example, loss-of-function mutations in Fas or Fas ligand (FasL) in mice and humans result in the overproduction of activated lymphocytes that ultimately trigger autoimmune syndromes. Further, cytotoxic T-lymphocytes express high levels of FasL, which induce the death of target cells that express Fas. This scenario is, however, exploited in cancer, where many tumor types have been shown to express elevated levels of FasL and thus to kill immune cells, thwarting this important mechanism of immune surveillance. Nonetheless, FasL and particularly TRAIL induce apoptosis of many cancer cell types, and TRAIL, as well as antibodies that cluster TRAIL receptors, induce tumor regression *in vivo*, underscoring their potential as therapeutics for cancer (19).

Tumor cells have developed an array of mechanisms that avert or disable apoptosis that is induced by the activation of death receptors. First, Fas is inactivated through somatic mutations in a number of human tumors (20). Second, Fas expression is suppressed in leukemias and neuroblastomas that fail to respond to front-line therapeutics, suggesting a role for the Fas pathway in some forms of drug resistance (21). Similarly, the expression of the pro-apoptotic TRAIL receptors, TRAIL-R1 and R2, is compromised in some tumor types through deletion or promoter hypermethylation. Third, several cancers overexpress soluble forms of death receptor ligands that inhibit rather than activate these receptors (22). Finally, some cancers gain protection by up-regulating the expression of TRAIL “decoy” receptors (TRAIL-R3 and TRAIL-R4) that essentially function as sinks for death ligands, as they fail to transmit a death signal due to loss of the entire cytoplasmic domain (R3) or to a truncated DD (R4) (81).

Nuclear Factor- κ B Family

Several signaling pathways converge on the apoptotic machinery to regulate cell survival. One target is the nuclear factor- κ B (NF- κ B) family of dimeric transcription factors that share a conserved N-terminal DNA binding/dimerization motif termed the “Rel domain.” The founding member of this family, v-Rel, was discovered in the viral genome of the Rev-T retrovirus, which causes the rapid development of lymphoma in young chickens (23). The mammalian NF- κ B family consists of the Rel proteins (RelA [p65], RelB, and c-Rel) and the NF- κ B proteins p50/p105 (NF- κ B1) and p52/100 (NF- κ B2), which have the ability to form homodimers or heterodimers and regulate gene expression in response to a wide array of stimuli (24). NF- κ B family members are normally held in an inactive state in the cytoplasm through their association with the inhibitors of NF- κ B, I κ B α or I κ B β (24). In response to specific stimuli, I κ B becomes phosphorylated by the I κ B kinase (IKK) signaling complex. Phosphorylated I κ B is then targeted

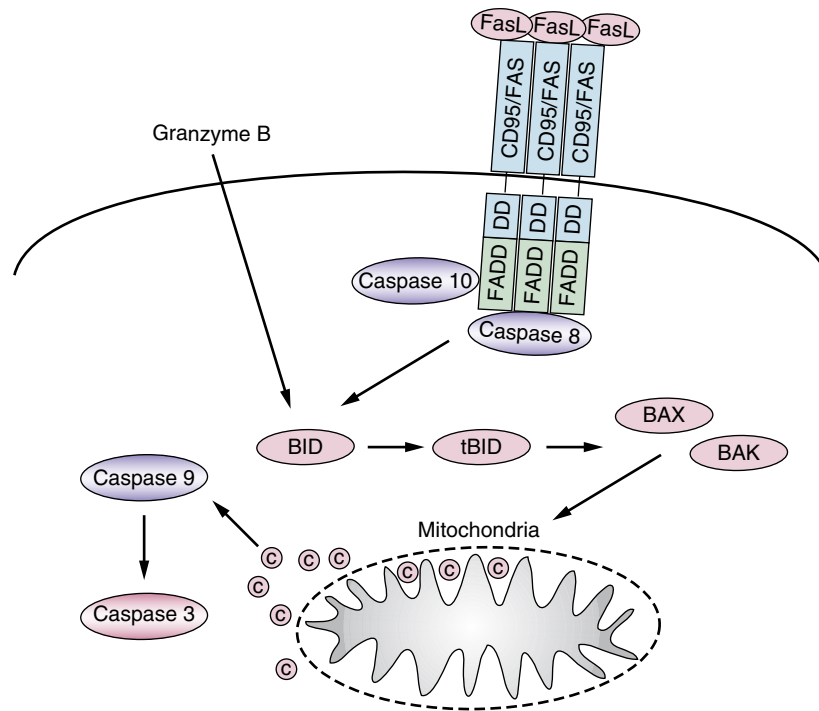


FIGURE 15-5 LINKING THE INTRINSIC AND EXTRINSIC CELL DEATH PATHWAYS. The intrinsic and extrinsic pathways are linked by the BH₃-only family member BID, which is activated through caspase-8 and/or granzyme B-mediated cleavage. Truncated BID, tBID, then binds to and facilitates the recruitment, oligomerization and activation of BAX and BAK, which together are required for all forms of apoptosis, at the mitochondrial outer membrane. In turn, activation of BAX/BAK induces mitochondrial membrane permeabilization (MOMP), releasing cytochrome *c* (small red circles) thus activating caspase-9 and its downstream targets such as caspase-3.

for destruction by the proteasome, releasing NF- κ B and allowing its transport to the nucleus where it regulates its target genes, some of which play key roles in regulating apoptosis (23).

NF- κ B transcription factors play essential roles in tumor development, including control of tumor angiogenesis, proliferation, inflammation, metastasis, differentiation, and survival (23). Importantly, the inactivation of several NF- κ B family members in mice has demonstrated their essential roles in the development of the immune system and in controlling cell survival. Conversely, the amplification of *c-Rel*, and/or mutations or deletions in I κ B that lead to constitutive activation of NF- κ B have been reported in several types of cancer, and NF- κ B induces the expression of several anti-apoptotic genes including Bcl-2, Bcl-X_L, A1, FLIP, Bfl-1, and the IAP family member *c-IAP2* (25). Thus, constitutive NF- κ B activation inhibits the intrinsic apoptotic pathway by targeting the Bcl-2 family, the extrinsic pathway by elevating levels of FLIP, and both by inducing IAPs that inhibit effector caspases. The prosurvival functions of NF- κ B have been further underscored by many studies that have demonstrated that inhibiting NF- κ B activity induces apoptosis in many cancer cell lines. Importantly, the anticancer effects seen with the proteasome inhibitor velcade (Bortezomib) may be in part attributed to inhibiting the degradation of I κ B, which in turn sequesters and inhibits NF- κ B. Indeed, targeting NF- κ B is an attractive arena in therapeutics as the inhibition of NF- κ B activity with thalidomide, nonsteroidal anti-inflammatory drugs, arsenic, curcumin, parthenolide, and small molecule inhibitors of IKK have shown potent antitumor activity.

Apoptosis-Inducing Factor

The mitochondria plays an important role in mediating the apoptotic response, as mitochondrial outer membrane permeabilization (MOMP) results in the release of a plethora of molecules that regulate the caspase cascade such as cytochrome *c*, XIAP, and Smac/DIABLO. In addition, apoptosis-inducing factor, AIF, which is normally localized to the inter membrane space of the mitochondria, is released from the mitochondria during apoptosis (reviewed in [26]). Knock-out studies indicate that AIF functions as an NADH oxidase necessary for optimal oxidative phosphorylation and that it plays a role in defense against oxidative stress. The *Harlequin* (*Hq*) mouse strain contains a retroviral insertion that results in an 80% to 90% reduction in AIF, and these mice suffer from neurodegeneration, skeletal muscle atrophy, and dilated cardiomyopathy, suggesting that AIF functions as a prosurvival molecule.

Although mitochondrial AIF promotes energy metabolism and protects from oxidative stress, during MOMP, AIF translocates to the nucleus where it can induce chromatin condensation, DNA strand breaks, and in some circumstances, cell death. The overexpression of AIF in HeLa cells results in the nuclear manifestations of apoptosis; however, AIF does not induce DNA damage on its own, but requires cyclophilin A to form an active DNase, which cleaves chromatin into large segments. Embryonic stem (ES) cells lacking AIF are resistant to serum withdrawal-induced cell death, yet they remain sensitive to etoposide and other apoptotic stimuli, suggesting that AIF is not required for apoptotic

cell death, but may be an important part of the machinery that dismantles the cell during apoptosis. Thus, while AIF's ability to induce apoptosis is under debate, AIF's roles in oxidative metabolism and mitochondrial bioenergetics suggest that its functions may help either to tip the balance toward cell death or survival. Indeed, cell death induced by DNA alkylating agents that activate poly(ADP-ribose) polymerase-1 (PARP-1), which generates large poly(ADP-ribose) (PAR) polymers, is dependent on AIF. PARP-1 requires NAD⁺ as a cofactor and its activation results in rapid glycolytic failure through depletion of cytosolic NAD⁺, and recent evidence suggests that PAR oligomers also provoke mitochondrial release of AIF, in a fashion independent of Bcl-2 family members, to induce cell death.

AKT/P TEN

The protein kinase AKT, also known as PKB, plays a vital role in growth factor signaling, and when constitutively activated AKT acts as an oncogene that drives cell growth and metabolism in the absence of growth factors (27). AKT was first discovered as a viral oncogene in the transforming retrovirus AKT8 (28). There are three AKT genes in humans and all are regulated by the production of the phosphatidylinositol-3,4,5-trisphosphate (PIP3) second messenger, which is generated by class I phosphoinositide-3-kinase (PI3K; 27). AKT is recruited to the plasma membrane through a pleckstrin homology (PH) domain, which binds to PIP3. Once at the membrane, AKT becomes phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK1) and PDK2.

AKT phosphorylates a number of substrates to inhibit apoptosis. First, AKT phosphorylates and inactivates the forkhead (FOXO) family of transcription factors, blocking their ability to induce pro-apoptotic regulators such as Bad and Fas ligand, and others (29). Similarly, AKT has been reported to phosphorylate and inactivate caspase-9, as well as I κ B; the latter resulting in the activation of NF- κ B, which inhibits apoptosis. AKT phosphorylates and stabilizes XIAP, which inhibits caspases, and AKT-mediated phosphorylation of Bax has been reported to inhibit its conformational change, which is necessary for its insertion into the outer membrane of mitochondria. Finally, AKT also phosphorylates and activates MDM2, which cancels p53's transcription functions and initiates p53 destruction, effectively disabling p53-dependent apoptotic pathways.

The activation of AKT in cancer can occur through amplification or somatic gain-of-function missense mutations of *PIK3CA*, which encodes the p110 catalytic subunit of PI3K, and through amplifications of AKT. Furthermore, inactivating mutations of *PTEN* (phosphatase and tensin homologue deleted on chromosome 10), a phosphoinositide phosphatase that negatively regulates AKT activation through the dephosphorylation of PIP3 at the plasma membrane, is one of the most widely mutated tumor suppressors in human cancers, and *PTEN*-deficient mice are tumor prone. Disruption of *PTEN* activity or the constitutive activation of AKT through somatic mutations or amplifications of *PIK3CA* or AKT renders tumors resistant to apoptosis induced by several chemotherapeutic agents, and in such scenarios AKT kinase inhibitors may restore the apoptotic program in cancer (29).

Oncogenic Stress and p53

Checkpoints that exist in the cell coordinately function to limit inappropriate cell proliferation and cell survival, and collectively block cell transformation. A key checkpoint is the p53 tumor suppressor pathway that controls cell fate by inducing cell cycle arrest or apoptosis. p53 functions as a transcription factor that responds to a wide variety of stimuli that induce cell stress, and in the context of activation of oncogenes such as *Myc*, *E1A* or *E2f1*, which induce cell cycle entry and provoke a hyperproliferative response, p53 becomes stabilized and activates transcription targets that collectively hold tumorigenesis in check. Accordingly, tumor cells often undergo mutations that directly result in loss-of-function mutations in p53, including missense mutations that generate dominant-negative forms of p53, epigenetic silencing, or biallelic deletions of p53.

Activation of the p53 pathway is a hallmark of malignancies that are provoked by the *Myc* family of oncogenic transcription factors, which are overexpressed in $\approx 70\%$ of all human cancers and which function as master regulators of cell growth and division (reviewed in [30]). Through an undefined mechanism, the stress induced by *Myc* overexpression activates the p53 pathway through the induction of the p19^{Arf} protein (p14^{ARF} in humans) that is encoded by the alternative reading frame of the *In4a* locus, *Arf* (reviewed in [31]). In turn, *Arf* activates p53 indirectly, by binding to and inactivating the functions of p53's endogenous inhibitor Mdm2, which itself is a p53 transcription target that holds the p53 response in check by binding to p53 and inhibiting its transcription functions. In addition, Mdm2 functions as an E3 ubiquitin ligase that ubiquitinates p53, which leads to its destruction by the proteasome. *Arf* blocks the E3 ubiquitin ligase activity of Mdm2 and thus in the presence of *Myc*, high levels of *Arf* lead to a sustained and robust p53 response that induces apoptosis. Accordingly, loss of *Arf* or p53 markedly accelerates *Myc*-driven tumorigenesis, whereas loss of *Mdm2* compromises this process, by inducing a massive, p53-dependent, apoptotic response. Importantly, mutations in components of this pathway, including its upstream regulators and downstream targets, are a hallmark of all malignancies.

Precisely how p53 induces apoptosis is contested (review in [32]). On one level, its activation or repression of key transcription targets drives cells to commit suicide. For example, p53 activates the expression of Puma or Noxa, BH3-only Bcl-2 family members that bind to and sequester anti-apoptotic proteins such as Bcl-2, allowing activation of Bax and Bak and apoptosis. Furthermore, p53 has been reported to also activate the transcription of Bax and of Apaf-1, a key component of the apoptosome that is activated by cytochrome *c* released from mitochondria and triggers self-cleavage and activation of caspase-9. Furthermore, regulation of the extrinsic cell death pathway by p53 also comes into play, where it activates the expression of the DR5 receptor for TRAIL and induces the expression of Fas ligand.

Although p53's transcription functions are an important mechanism for induction of apoptosis, other more provocative functions for this tumor suppressor protein have also been

described. In particular, during activation p53 also accumulates in the cytoplasm and here it has been shown to associate directly with mitochondria, and to regulate apoptosis at this site (32). Indeed, targeting p53 directly to the mitochondria induces MOMP, which is inhibited by the overexpression of Bcl-2 or Bcl-X_L. Interestingly, both Bcl-2 and Bcl-X_L bind to p53 at the mitochondria, suggesting that they play a role in p53 localization to the mitochondria. Moreover, recent evidence suggests that Puma displaces p53 from the Bcl-X_L/p53 complex at mitochondria, allowing p53 to induce MOMP and apoptosis. The mechanism by which p53 induces MOMP in cells is not clear, but the addition of p53 to purified mitochondria can induce MOMP, and p53, similar to tBid, can induce the oligomerization of purified Bax. Although evidence is mounting that cytoplasmic p53 indeed plays important roles in the apoptotic program, it seems likely that this works in concert with p53-dependent transcriptional responses to provoke efficient and rapid cell suicide.

Autophagy

Autophagy translates as “self-eating” and is simply defined as the delivery to and degradation of cytosolic material and organelles by the lysosome. The lysosome, initially characterized in 1955 by Christian de Duve as a membrane-bound compartment containing acid phosphatases, is a degradative organelle. Lysosomes are acidic and contain acid hydrolases, nucleases, peptidases, proteases, phosphatases, sulfatases, glycosidases, and lipases, which together are capable of dismantling all macromolecules present within the cell. Degradation of substrates by these enzymes

recycle macromolecules for reuse in biosynthetic processes, yet, when unrestrained autophagic degradation can also lead to the annihilation of the cell.

Rather than one discrete mechanism, autophagy represents a collection of processes, which are differentiated by the routes in which cytosolic material is delivered to the lysosome. Microautophagy is characterized by the direct invagination of the lysosomal membrane resulting in a vesicle that contains cytosolic material, which is subsequently degraded (Figure 15-6; 33). In contrast, chaperone-mediated autophagy targets proteins to the lysosome via targeting sequences that consist of the short-consensus peptide sequence, KERFQ. This peptide sequence is recognized by the cytosolic chaperone hsc73, which targets proteins to the lamp2a receptor on the lysosomal membrane (Figure 15-6; 33). Proteins are unfolded by cytosolic hsc73, and with the aid of lysosomal hsc73, they are transported across the lysosomal membrane and are then degraded.

Macroautophagy, hereafter referred to as autophagy, was originally described as a cell death mechanism morphologically distinct from apoptosis (Figure 15-1; 34). Autophagy is the more ancient program of the two, as it is evolutionarily conserved from yeast to human and it plays dual roles in both cell death and survival. Indeed, autophagy is the major mechanism for degrading long-lived proteins, organelles, and large protein complexes (35,36). The autophagosome nucleates from flat cisternae of membranes called the phagophore (Figure 15-6). Although the origin of this membrane is uncertain, early autophagosomes contain markers of rough endoplasmic reticulum. Double-membraned autophagosomes envelop organelles and cytoplasmic materials; this process can occur in bulk or through targeting specific cargo (37).

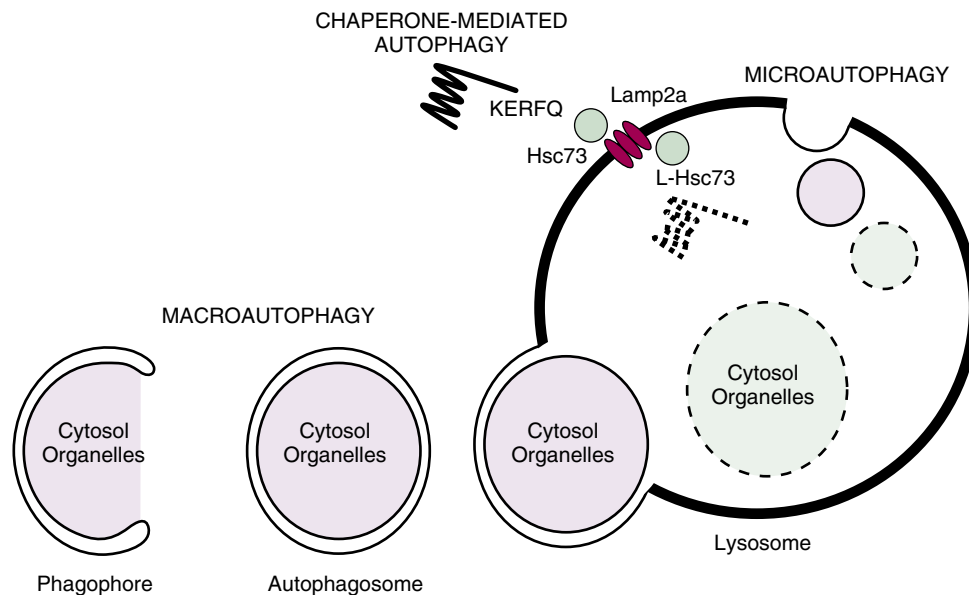


FIGURE 15-6 AUTOPHAGY IS LYSOSOME-MEDIATED DESTRUCTION. Autophagy is the delivery of cytosolic material to the lysosome for degradation/recycling. Three major pathways for lysosomal delivery are known and as a result are separated into three classes of autophagy. Microautophagy is the direct invagination of the lysosomal membrane, which engulfs cytosolic material resulting in a vesicle that pinches into the lumen of the lysosome and is subsequently degraded. Chaperone-mediated autophagy is the direct targeting of proteins via a *cis*-peptide sequence (KERFQ) by the chaperone Hsc73, which then unfolds and translocates the protein into the lumen of the lysosome for degradation by Lamp2a and Hsc73 in the lumen of the lysosome. Macroautophagy results from the formation of a double-membrane vesicle (autophagosome) that can engulf both bulk cytoplasm and organelles such as mitochondria. Once formed the outer membrane of the autophagosome then fuses with the lysosome delivering the inner vesicle and its contents for degradation.

Autophagy was originally defined as a cell death mechanism (35), yet studies in yeast have clearly shown that autophagy is a survival response during times of starvation, a condition highly germane to the tumor microenvironment. The identification of the first genes required for autophagy came from a screen in *Saccharomyces cerevisiae* that identified mutants that failed to accumulate autophagic bodies in response to nitrogen starvation. In total, 15 genes were shown to be required for autophagy (*ATG1–ATG15*). Fourteen more autophagy-specific genes have since been discovered in yeast, bringing the total to 29 *ATG* genes. Although autophagy is conserved in man, to date less than ten mammalian homologues of these *ATG* genes have been identified.

The second functional group of ATG genes comprise the components of the PI3K complex, which consists of Atg6 (Beclin), Atg14, Vps15, and Vps34 (40). This complex regulates the formation of PI3P, which is necessary for forming autophagosomes. Together, the Atg1-kinase complex, and the PI3K complex activate the autophagy pathway. Interestingly, the mammalian homologue of Atg6, *Beclin-1*, can complement the autophagy defect in $\Delta atg6$ -deficient yeast (41), underscoring the remarkably conserved nature of this pathway. Importantly, PI3K inhibitors such as 3-methyladenine and wortmannin inhibit autophagy, but unlike *S. cerevisiae*, which has only one PI3K (Vps34), mammals have class I, II, and III PI3Ks. In mammals, *Beclin-1* is associated with the class III PI3K complex and

The last two functional groups of ATG genes, the Atg12 conjugation and Atg8 (LC3) conjugation systems, contribute to the actual formation of autophagic vesicles (reviewed in [42]). Both are ubiquitin-like systems that regulate and are necessary for vesicle formation (Figure 15-7). Atg12 and Atg8 are both ubiquitin-like proteins that are activated by a common E1-like enzyme, Atg7, through the formation of thioester intermediates. Atg7-activated Atg12 and Atg8 moieties are then transferred to the E2-like enzymes, Atg10 and Atg3, respectively. Atg10 subsequently directs the conjugation of Atg12 to Atg5. Similarly, Atg8 and its mammalian homologue LC3 are conjugated by Atg3, but unlike any other ubiquitin-like proteins, they are conjugated to the lipid phosphatidylethanolamine (PE). The conjugation of Atg8 (LC3) to PE generates Atg8-PE (LC3-II), which tightly associates with vesicle membranes. Importantly, the induction of autophagy absolutely correlates with the formation of lipidated forms of LC3 (42). Indeed, a GFP-LC3 fusion acts as an accurate reporter of autophagic vesicle formation. When autophagic activity is low, GFP-LC3 is diffuse in the cytosol, but when autophagy is activated (e.g., following nutrient starvation) GFP-LC3 rapidly redistributes to punctate autophagic vesicles (82).

FIGURE 15-7 **UBIQUITIN CONJUGATION PATHWAYS THAT REGULATE MACROAUTOPHAGY.** A system of two ubiquitin-like conjugation systems regulates the formation of autophagosomes. Both ATG12 and ATG8 (LC3) resemble ubiquitin (*red ovals*) and are activated for conjugation by the same E1, ATG7. After activation, these ubiquitin-like proteins are then transferred to their respective E2s, ATG10 and ATG3, and subsequently conjugated to their partners. While ATG12 is conjugated to the protein ATG5, unlike all other ubiquitin-like molecules, ATG8 (LC3-I) is conjugated to the lipid phosphatidylethanolamine (PE) generating LC3-II. The formation of both of these conjugates is required for the formation of autophagosomes. Although most ubiquitin-based pathways require an E3 ligase, no such ligase has been identified thus far for the generation of either the atg 5-12 conjugate, or LC3-II.

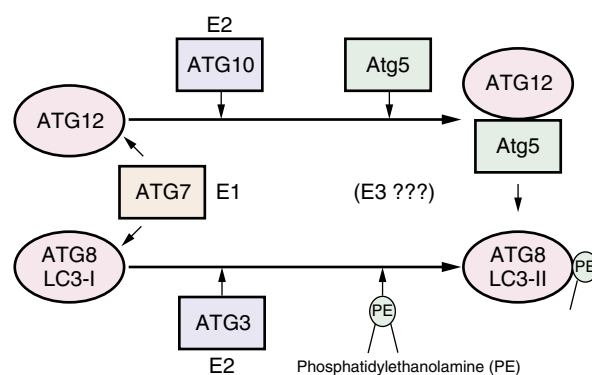


FIGURE 15-7 UBIQUITIN CONJUGATION PATHWAYS THAT REGULATE MACROAUTOPHAGY. A system of two ubiquitin-like conjugation systems regulates the formation of autophagosomes. Both ATG12 and ATG8 (LC3) resemble ubiquitin (*red ovals*) and are activated for conjugation by the same E1, ATG7. After activation, these ubiquitin-like proteins are then transferred to their respective E2s, ATG10 and ATG3, and subsequently conjugated to their partners. While ATG12 is conjugated to the protein ATG5, unlike all other ubiquitin-like molecules, ATG8 (LC3-I) is conjugated to the lipid phosphatidylethanolamine (PE) generating LC3-II. The formation of both of these conjugates is required for the formation of autophagosomes. Although most ubiquitin-based pathways require an E3 ligase, no such ligase has been identified thus far for the generation of either the atg5-12 conjugate, or LC3-II.

chemotherapeutic agents seem to induce the protective affects of autophagy in cancer cells contributing to chemoresistance (45). Thus, the formation of autophagic vesicles has been associated with cell death and cell survival in cancer. Early studies suggested that autophagy is repressed during cellular transformation, yet genetic evidence that this is the case has only recently been uncovered (50).

The first direct genetic links between cancer and autophagy came with the discovery that Beclin-1 interacts with the anti-apoptotic oncoprotein Bcl-2 (reviewed in [46]). Furthermore, similar to Bcl-2, Beclin overexpression inhibits neuronal cell death in response to virus infection. These data suggested that Beclin-1 would function as an oncogene similar to Bcl-2. However, *Beclin-1* resides on 17q21, a hot spot for chromosomal deletions in human cancer, and indeed deletion of one allele of *Beclin-1* has been reported for a large number of spontaneous breast and ovarian cancers, suggesting that Beclin functions as a tumor suppressor. In support of this notion, the generation of *beclin-1* knock-out mice revealed that while *beclin-1*^{-/-} embryos die at day E7.5, *beclin-1*^{+/-} mice are tumor prone (to hepatocellular and lung carcinoma and B-cell lymphoma; latency $\approx$ 16–18 months of age) (50). Furthermore, studies using cancer cell lines have indicated that *Beclin-1* haploinsufficiency leads to defects in autophagic activity in response to amino acid starvation and that the activation of autophagy is associated with impaired tumorigenesis in xenografts. Therefore, autophagy may provide tumor suppressive functions.

One obvious mechanism for tumor suppression by autophagy would be the induction of autophagic cell death. For example, treatment of glioblastoma cells with arsenic trioxide induces cell death that morphologically resembles autophagy and is not inhibited by Bcl-2 overexpression or caspase inhibition (45). Rather, this cell death is inhibited by the V-type ATPase inhibitor bafilomycin A1, which blocks the acidification of lysosomes and thus autophagic degradation. Similarly, autophagic cell death in some cancer cell lines is inhibited by the autophagic inhibitor 3-methyladenine and has been observed in cells chronically treated with broad-spectrum caspase inhibitors. This autophagic death is prevented by siRNA-mediated knockdown of either *Atg6* or *atg7* (84). Collectively, such studies, along with those showing that *bax/bak*-deficient cells, which are defective for apoptosis, undergo *Atg5*- and *Atg7*-dependent cell death in response to staurosporine or etoposide treatment (48), indicate that autophagy can function as a cell death response that could potentially curtail tumor growth.

There are also intimate links between autophagy and apoptosis. Early observations indicated that protein synthesis was required for apoptosis and several autophagy proteins are known to be up-regulated during apoptosis. Indeed, the mammalian homologue of ATG5 was first identified as such a protein, and *Atg5* has been reported to associate with FADD, a component of the extrinsic apoptotic pathway (35). Interestingly, enforced expression of *Atg5* enhances the susceptibility of certain cancer cell lines to apoptosis, whereas siRNA-directed knockdown of *Atg5* confers resistance to several chemotherapeutic agents (83). One study has suggested more direct links. For example, *Atg5*

is cleaved by calpain following the induction of apoptosis, and then translocates from the cytosol to the mitochondria where it associates with Bcl-X_L, inducing the release of cytochrome *c* and activating the caspase cascade. Furthermore, in addition to interacting with Bcl-2, Beclin-1 has been reported to interact with the anti-apoptotic Bcl-X_L and Mcl-1 proteins, which, when overexpressed also inhibit autophagy in a Beclin-dependent manner (35). However, at odds with these observations are the findings that mouse embryo fibroblasts defective for Bax and Bak die can die in an autophagy gene-dependent manner in response to staurosporine and etoposide, and rather than inhibiting autophagy, Bcl-2 or Bcl-X_L overexpression sensitizes these cells to autophagic cell death (48). Therefore, although there are clear links between autophagy and apoptosis, the functional relevance of these interactions and their relevance to cancer cell responses to therapeutics are not yet resolved.

It is clear that autophagy is, on some level, a required pathway for tumor development and/or maintenance. Importantly, in tumors from both mice and humans, both alleles of *Beclin-1* are never cocomitantly detected, suggesting that the complete loss of autophagy may be selected against during tumorigenesis (46). Furthermore, because autophagy is activated as a proximal response to metabolic stress, it could play an important role in the clearance of damaged mitochondria, which would prevent apoptosis. Similarly, autophagy likely provides essential nutrients during times of hypoxia and starvation—conditions that are frequently encountered in the microenvironment of rapidly dividing tumors. Therefore, cancer cells may functionally reset the rheostat of autophagic activity to a level that is necessary for growth but inhibits autophagy-induced cell death. This would suggest that a low level of autophagy is beneficial for tumor growth. This notion is supported by observations that signals that impair autophagy, such as constitutively active AKT or haploinsufficiency of *Beclin-1*, when combined with the loss of pro-apoptotic regulators, provoke accelerated tumor growth (49). Such tumors display increased metastasis, indicating that a reduction in autophagy can actually lead to a more aggressive tumor phenotype (49).

Whether autophagy functions as a physiologic cell death mechanism is controversial, yet there is no doubt that this pathway plays an essential role in cell survival, which has been borne out by gene deletion studies in yeast and mice. For example, deletion of *beclin-1* in mice leads to mid-gestational embryonic lethality (50), whereas deletion of *Atg7* or *Atg5* leads to perinatal lethality due to the failure to activate the autophagy pathway during the starvation interval that immediately follows birth, before suckling can ensue (51,52). However, it is not difficult to imagine that autophagy can also lead to the eventual destruction of the cell, but here the mode of cell death, with all its resources and energy expended, is more likely to be necrotic than apoptotic cell death (see Necrosis). Regardless, it is clear that components of the autophagy pathway represent an untapped and fertile ground for new targets in chemotherapy, and it seems likely that drugs that modulate this pathway may prove to be effective agents for the prevention and treatment of cancer.

Necrosis

In addition to apoptosis and autophagic cell death, another, less well-characterized form of cell death exists: necrosis. Historically, cell death was thought to be an abnormal response and all forms were collectively described as necrotic, derived from the Greek *nekros*, for “corpse.” However, with the discovery and elucidation of apoptosis as a developmental and homeostatic requisite, PCD gained acceptance as part of normal physiology. Necrosis has traditionally been defined as uncontrolled, chaotic, and disordered process of cell destruction that has been viewed as “accidental,” or as a passive response to overwhelming physiologic extremes, such as hyperthermia, mechanical shear force, anoxia/ischemia, or exposure to certain toxins.

However, certain means of inducing necrosis suggest that it may represent a form of PCD, similar to apoptosis and autophagic cell death. Indeed, it is increasingly apparent that pathways leading to necrosis involve complex cellular and enzymatic machinery and that as such it may represent an ordered process. Necrosis ensues as the result of overwhelming bioenergetic failure. Specifically, this bioenergetic failure (i.e., lack of sufficient ATP to maintain cellular processes) is often preceded disruption of the plasma membrane, mitochondrial dysfunction, dysregulated calcium levels, supraphysiologic reactive oxygen species (ROS) production, and proteolysis mediated by calcium-dependent proteases (53). Many of these responses have all the hallmarks of a cellular “program” because they are enzyme dependent and involve the activation or inhibition of specific signal transduction pathways that can be influenced by both genetic and epigenetic factors (53).

Inhibition of apoptosis or autophagy in certain circumstances has been shown to lead to necrosis, implicating it as the “default” cell death pathway. For example, caspase inhibition in certain cell types leads to necrosis (54). In addition, if the developmental apoptotic cell death in the interdigital regions of the developing mouse embryo are inhibited, cell death still proceeds, but switches to a necrotic morphology (55). However, necrosis does not appear limited to developmental processes. In adult mice, haploinsufficiency of *beclin-1* or knock-down of *atg5*, coupled with the *bax/bak* deficiency, results in the induction of a necrotic-like death under stress conditions (49). A necrotic death pathway is also found in lower organisms such as yeast and protozoa, indicating that necrosis is evolutionarily conserved (56).

Several lines of evidence indicate that necrosis ensues after the induction of defined programs. First, necrosis is involved in a variety of developmental and homeostatic processes. For example, chondrocytes located in growth plate regions have been shown to turnover via necrotic, as well as apoptotic, mechanisms. Similarly, the targeted turnover of enterocytes and crypt cells in adult intestinal epithelium can involve necrosis (53). Data support a role for necrosis in maintenance and regulation of immune responses and in the physiology of wound repair (53,57). For example, T-lymphocytes undergoing negative selection during immune responses exhibit necrotic morphology, and this selection is independent of caspase activation (53,58). Thus, necrosis appears to be important in the regulation of adaptive immunity.

Necrosis is also a preferred mechanism of cellular elimination in some disease states. For example, vaccinia virus–infected Jurkat T cells undergo necrosis in response to tumor necrosis receptor (TNFR) signaling, and TNFR2-deficient mice are compromised in viral clearance that may be associated with defects in necrosis. Indeed, the inflammatory response that accompanies necrosis may be a necessary defense against invading pathogens, functioning as an alarm mechanism that activates the innate immune cascade (53,58).

Morphologically, necrosis involves plasma membrane rupture, coupled with a marked swelling of organelles, particularly mitochondria. Intracellular contents of necrotic cells then spill out into the extracellular milieu, inducing an inflammatory response in the surrounding tissue. This contrasts to other forms of cell death, such as apoptosis, in which plasma membrane integrity is maintained, allowing cellular contents to be neatly packaged into apoptotic bodies, which are readily cleared by phagocytes (59). However, if apoptotic cells are not cleared (such as during in vitro culture conditions), late-stage apoptotic cells will begin to show characteristic signs of necrosis, an event that has been termed “secondary necrosis.” This is presumably due to eventual bioenergetic exhaustion, but unfortunately has led to necrosis periodically being described merely, as a tissue culture artifact.

Receptor-Mediated Necrosis

Necrosis can be initiated in response to activation of the “death receptors” (e.g., TNFR1, Fas, and TRAIL), which also triggers apoptosis (see preceding sections). For example, FADD recruitment to TNFR1 has been demonstrated to induce necrosis in L929 fibrosarcoma cells (55), and inhibition of caspases does not prevent this necrotic cell death, but rather enhances it. Thus, certain signal transduction cascades appear to be specific for necrosis, rather than being merely co-opted from other forms of cell death. The ultimate effector of necrosis in this model is a high level of ROS, as necrosis can be blocked by the oxygen radical scavenger butylated hydroxyanisole (BHA; 60). Interestingly, when Fas expression is enforced in L929 cells and the receptor is triggered, both apoptotic and necrotic programs proceed concurrently, and if apoptosis is blocked, necrosis is unaffected (61).

Similar to FADD, the adaptor serine/threonine kinase RIP1 has also been shown to play a role in mediating necrosis (58). Indeed, both FADD- and RIP1-deficient T cells are resistant to necrotic death induced by TNF- α or Fas ligand (62). RIP1 involvement in necrosis is not fully understood, but there are several potential mechanisms. Unlike their wild-type counterparts, RIP1-deficient Jurkat cells fail to accumulate ceramide after TNF- α treatment, and ceramide accumulation also leads to necrosis in a TRAIL-mediated model of caspase-independent PCD. RIP1 has also been implicated in necrosis that is induced following alkylation of DNA and subsequent PARP activation (63). At a mechanistic level, RIP1 may affect this pathway by virtue of its ability to disrupt proper ATP balance, through its suppression of the adenine nucleotide translocase (ANT) enzyme that is present in the outer membrane of mitochondria, leading to bioenergetic failure and necrosis (64). However, like many regulators of cell death

pathways, RIP1 likely also has functions in other forms of cell death, including induction of autophagy (53).

In addition to necrosis directed by death receptors, several types of excitoreceptors expressed by neuronal cells can also trigger necrosis, depending on the intensity of the stimulus they transmit (55). For example, depletion of ATP pools following ischemia/anoxia or hypoglycemia results in the loss of neuronal plasma membrane integrity and the release of excitatory neurotransmitters from presynaptic neurons. Glutamate receptors, including *N*-methyl-D-aspartate (NMDA) and β -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors, induce Ca^{2+} overload in the postsynaptic neurons, which in turn activates calpains, which are calcium-dependent cysteine proteases. Activated calpains then provoke a feed-forward loop via their cleavage of the $\text{Na}^+/\text{Ca}^{2+}$ exchange channel, which causes prolonged elevation of intracellular Ca^{2+} and eventual necrotic death. This response is also dependent on the metabolic status of mitochondria. Finally, purinergic receptors also trigger necrosis by binding to exogenous stores of ATP, a response that induces pore formation in the plasma membrane, influx of Ca^{2+} , depolarization of membranes and necrosis.

Ca^{2+} and Oxidative Stress Provoke Necrosis

Potent increases in intracellular Ca^{2+} trigger cell death. Generally, it is thought that Ca^{2+} levels need to be in the micromolar range to induce necrosis, whereas lower levels preferentially induce apoptosis. Micromolar Ca^{2+} levels are consistent with plasma membrane depolarization, rather than that provoked by the release of Ca^{2+} stores from the ER, which is associated with apoptosis. Chelation of extracellular Ca^{2+} (EGTA, BAPTA) prevents hypoxia- and hypoglycemia-induced necrosis (55). High levels of Ca^{2+} activate calpains, stimulate increased ROS generation by mitochondria, and induce mitochondrial permeability transition (mPT). In turn, this results in the loss of mitochondrial membrane potential, mitochondrial swelling, and the abolishment of ATP synthesis via oxidative phosphorylation (65), all hallmarks of necrosis. This pathway appears to be caspase independent, but is dependent on expression of cyclophilin D (CypD), and can be blocked by cyclosporin A, a peptidyl-prolyl isomerase family inhibitor. Indeed, CypD-deficient mice are resistant to necrosis induced by Ca^{2+} flux and oxidative stress (66) and exhibit greatly reduced damage in ischemia/reperfusion cardiac injury models. Accordingly, CypD transgenic mice show increased susceptibility to mPT, and a CypD-overexpressing neuronal cell line favors necrosis while inhibiting apoptosis (67). The mechanisms surrounding CypD and induction of mPT are unknown (65). Regardless, CypD is revealed as a critical regulator of necrosis, which appears to be an important mechanism of cell death in both physiologic and pathophysiologic scenarios.

Poly(ADP-Ribose) Polymerase: Critical Arbiter of Necrosis

Induction of necrosis is dependent on the energy balance of the cell and depletion of ATP beyond tolerable limits invariably leads to necrotic cell death. Although loss of ATP may result from extracellular damage, intracellular insults, particularly those invoking

DNA damage, also drain the cellular energy pool. Damage to DNA (single- or double-stranded breaks) activates a repair pathway involving the poly(ADP-ribose) polymerase (PARP) family of proteins. PARP-1 catalyzes the addition of ADP-ribose oligomers to DNA-binding proteins (e.g., to histones) at regions harboring DNA strand breaks (68). These modifications open up chromatin in the damaged region, facilitating access of DNA repair enzymes. ADP-ribose polymers are derived from NAD^+ , and exhaustion of cytosolic NAD^+ leads to a concomitant depletion of ATP by inhibiting glycolysis (55). Thus, if DNA damage is extensive (i.e., due to DNA alkylation, ischemia/reperfusion injury, or excitotoxicity), the loss of ATP becomes so profound that necrosis ensues. Not surprisingly then, mice deficient in PARP-1 are protected from ischemia/reperfusion injury, and inhibition of PARP-1, either chemically or by RNAi knock-down, suppresses necrosis (63) as does NAD^+ supplementation. Finally, PARP-1 also contributes to mitochondrial dysfunction during necrosis, mediating the release of AIF, a process that appears to rely on TRAF2/RIP1-dependent activation of JNK (69).

Necrosis and Cancer

Given its recently revealed wiring, mediators of necrosis (e.g., PARP-1 or its regulators, calpains and CypD) may provide new targets for cancer prevention or treatment. Traditionally much attention has focused on the induction of apoptosis in cancer therapy. However, during transformation most cancer cells acquire gain- or loss-of-function mutations that confer resistance to apoptosis, rendering intervention in this death pathway ineffective. Thus, cell death pathways such as autophagy and programmed necrosis may provide important alternative therapeutic targets. This is particularly germane to solid malignancies, whose central regions are often necrotic due to chronic hypoxia and nutrient depletion, a fact that also indicates that tumor cells are not inherently resistant to this form of cell death. Indeed, strategies targeting PARP-1 have been considered (70), as have studies linking the effects of therapeutics to both calpain-dependent tumor cell necrosis and resistance.

Exploiting Necrosis in Antitumor Immunity

Necrotic cells provoke strong inflammatory responses, suggesting that strategies that induce necrosis in neoplastic cells might augment existing antitumor defenses (54). Necrotic by-product and recruits activates a variety of immune cells, including neutrophils, dendritic cells, and macrophages, with the latter two serving as professional antigen-presenting cells (APCs). Unlike apoptosis, which is immunologically discreet, necrosis is accompanied by release of many pro-inflammatory mediators (54,71). Here, proteins such as heat shock proteins (hsp's) 70 and hsp90, high-mobility group box protein-1 (HMGB-1), calreticulin, as well as nucleosomes and RNA, potentiate activation of innate and adaptive immune cascades (54). For example, HMGB-1 enhances cytokine secretion from macrophages (72) and binds to extracellular receptors, including Toll-like receptors (TLRs) and the receptor for advanced glycated end products (RAGE). Further, APCs are activated by many of these inflammatory molecules (e.g., Hsps,

RNA, and calreticulin). For example, RNA binds to and activates TLR3, which then activates dendritic cells. Maturation of APCs in turn augments adaptive immunity through enhanced lymphocyte recruitment and activation. For example, *in vivo* gancyclovir treatment of thymidine kinase-expressing mouse melanoma tumors leads to tumor necrosis rather than apoptosis, and this is accompanied by pronounced infiltration of both activated macrophages, and T_H1 type T cells.

Therapeutic strategies based on the notion that necrosis is a default death pathway when apoptosis is blocked can also be envisioned. For example, preventing the timely clearance of apoptotic cells and apoptotic bodies may lead to secondary necrosis and inflammation. Here compounds that “mimic” apoptotic cells would competitively inhibit the uptake of apoptotic bodies by phagocytes, allowing these targets to then become necrotic. Indeed, phosphatidylserine-impregnated liposomes have been reported to inhibit the phagocytosis of apoptotic cells by human monocytes (73). Thus, therapies that induce necrosis may be desirable, not only for direct antitumor effects, but also for the accompanying immune activation that marshals the defenses of the host.

Chemotherapeutic Strategies to Target Necrosis

The mechanism of action for cancer drugs depends not only on the drug, but also on cancer type, the environmental milieu, and other factors, such as timing or dosage. Although certain chemotherapeutic drugs function through the induction of apoptosis or mitotic arrest, such as paclitaxel (74), others such as docetaxel, preferentially induce necrosis (75). Some compounds are known to induce both apoptosis and necrosis, such as cisplatin, β -lapachone, doxorubicin, and imatinib mesylate (Gleevec; 76–78). In general, higher dosages appear to favor necrosis over apoptosis. Tailoring drug regimens to trigger necrosis may be an important strategy in scenarios where resistance to apoptotic stimuli can be anticipated (e.g., tumors bearing loss-of-function mutations in p53 or that overexpress Bcl-2) or under unfavorable conditions (i.e., hypoxia; 79). For example, *N*-(4-hydroxy-phenyl) retinamide (4-HPR) induces necrosis in neuroblastoma tumor cell lines under conditions of low oxygen, is not affected by p53 status, and functions even in the presence of pan-caspase inhibitors. Switching the primary mechanism of killing to necrosis

over apoptosis may also be as simple as modifying the composition of the treatment. For instance, hydroxypropylmethacrylamide (HPMA) copolymer-conjugated doxorubicin preferentially induces necrosis in human ovarian carcinoma cells, presumably due to accelerated effects on plasma membrane permeability as compared to free doxorubicin.

Photodynamic Treatment and Necrosis

Photodynamic treatment (PDT) involves chemical sensitization of tumor cells with compounds that are activated following exposure to defined wavelengths of light (reviewed in [80]). These compounds, termed “photosensitizers,” are administered systemically, but are preferentially retained in tumor cells. Dependent on the photosensitizer, specific light spectra are administered to the tumor through the skin or via endoscopic delivery. Photoactivation generates reactive oxygen radicals, most of which are fatally reactive, singlet oxygen species. Death ensues directly and involves both apoptosis and necrosis. Damage to surrounding tissue can also contribute due to hypoxia that follows the loss of supporting vasculature. At the cellular level, damage to the plasma membrane plays a major role, and other membranes are targeted as well, including those of lysosomes and mitochondria. PDT therapy also generates strong immune responses against tumor cells, which may aid in tumor clearance.

Conclusion

Although it is clear that unique triggers and signaling pathways control the activation and execution of apoptosis, autophagy, and necrosis, it is increasingly evident that there are significant levels of cross-talk between the three pathways. It is likely that aspects of these three pathways are involved in the demise of the cell in response to signals or stresses, and that the end result represents a continuum of the integration of all forms of cell death. Thus, strategies focused on one without taking the others into account are likely to fail, as individual pathways are frequently defective in cancer. In contrast, combinatorial strategies that target all three pathways simultaneously are likely to be highly effective and should be the focus of future therapeutic intervention.

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Cellular Senescence

More than four decades ago, Hayflick and colleagues formally demonstrated that normal human cells—in this case, fibroblasts from fetal and adult tissue—have only a limited ability to proliferate in culture (1). These classic studies showed that cells from tissue explants initially underwent robust cell division in culture, but, gradually and progressively with each passage, the cultures accumulated viable nondividing cells. Eventually, all cells in the culture failed to proliferate, despite optimal growth conditions. This decline in cell division capacity was termed “cellular senescence” because it was proposed to recapitulate aspects of organismal aging. However, Hayflick and colleagues also noted that tumor cells behaved very differently: In contrast with normal human cells, tumor cells proliferated indefinitely in culture. This observation spawned the idea that cellular senescence might also suppress the development of cancer.

Forty years later, the hypothesis that cellular senescence contributes to organismal aging remains a viable but still tenuous possibility (2–5). In contrast, multiple lines of evidence now support the idea that cellular senescence suppresses carcinogenesis (6–11). Furthermore, the apparently disparate roles that have been proposed for cellular senescence (aging and tumor suppression) are beginning to converge.

Cellular Senescence: Characteristics

The salient feature of cellular senescence is an essentially irreversible arrest of cell proliferation (used here interchangeably with growth). In essence, the senescence response converts a mitotic cell, which has the ability to proliferate, into a postmitotic cell, which has permanently lost the ability to divide. Because cell proliferation is essential for tumorigenesis (7) the senescence growth arrest is undoubtedly the key feature that suppresses the development of cancer.

Cellular senescence is distinct from quiescence (reversible growth arrest), but at least with regard to loss of proliferative capacity resembles terminal differentiation. Indeed, the mechanisms that permanently arrest the growth of terminally differentiated and senescent cells may overlap. However, terminal differentiation is effected by developmental programs that ensure tissue maturation and function, whereas the senescence response is triggered by stimuli, insults, or stresses that put mitotic cells at risk for malignant transformation (11–14).

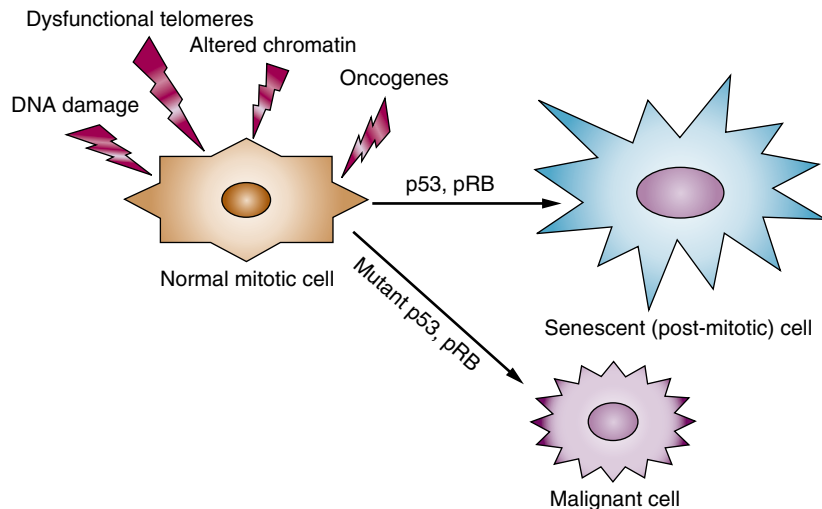
The senescence growth arrest is not simply a cessation of cell division. Rather, the senescence response entails widespread changes in gene expression, only some of which account for the loss of cell division capacity (15–20). A common characteristic of senescent cells is an up-regulation of genes encoding secreted molecules that can alter the tissue microenvironment. As discussed later, this feature of the senescence response may be the consequence of an evolutionary trade-off between tumor suppression and longevity and may even, ironically, fuel the development of late-life cancer (21).

Cellular Senescence: Causes

It took nearly three decades after Hayflick and colleagues described cellular senescence to understand that telomere shortening was a prime cause for the observed decline in cell proliferation. Telomeres are the repetitive DNA sequence (TTAGGG in vertebrates) and specialized proteins that form a protective “capped” structure at the ends of linear chromosomes (22). The telomeric structure prevents cellular DNA repair machineries from recognizing chromosome ends as broken DNA and thus prevents aberrant chromosome fusions and subsequent genomic instability (23). Because DNA replication is bidirectional and carried out by polymerases that are unidirectional and dependent on preexisting (labile RNA) primers, the telomeres cannot be completely replicated during DNA synthesis, a phenomenon termed the “end-replication problem.”

The end-replication problem can be overcome by telomerase, the enzyme that can add telomeric DNA repeats to the chromosome ends *de novo* (24). Among normal human cells, telomerase expression is confined to early embryonic cells, germ cells, and possibly some somatic stem or progenitor cells (25,26). Consequently, the telomeres shorten with each round of DNA replication in most human cells (27). Eventually, the telomeres become critically short and fail to form the protective capped structure (28). Dysfunctional telomeres cause genomic instability, which in turn leads to malignant transformation; consistent with cellular senescence being tumor suppressive, dysfunctional telomeres trigger a senescence response (29,30). Consistent with this view, ectopic expression of telomerase prevents telomere shortening and the decline in cell proliferation observed by Hayflick and colleagues (31). Telomerase expression *per se* does not cause neoplastic transformation (32).

FIGURE 16-1 Diverse oncogenic insults induce cellular senescence. Normal cells that have the ability to proliferate (mitotic cells) can be induced to undergo cellular senescence by potentially oncogenic insults, including DNA damage, dysfunctional telomeres, chromatin perturbations, and the expression of certain oncogenes. The senescence response permanently suppresses cell proliferation, effectively implementing a postmitotic growth arrest. Cells that lack functions of the p53 or pRB tumor-suppressor pathways are deficient in undergoing senescence. When faced with potentially oncogenic insults, such cells are at greatly increased risk for malignant transformation.



However, most cancer cells overcome the end-replication problem by activating telomerase expression (33,34).

It is now clear that dysfunctional telomeres are but one of many stimuli that can elicit a senescence response (Figure 16-1). Other senescence inducers include severe or irreparable DNA damage, including DNA double-strand breaks and damage caused by oxidative stress (35,36). In fact, dysfunctional telomeres are now thought to induce senescence by triggering a DNA damage response similar to that caused by DNA double-strand breaks (37,38). In addition, agents or genetic manipulations that perturb chromatin structure can cause cellular senescence, as can ill-defined stresses such as suboptimal growth conditions (39–43). Furthermore, intense or unbalanced mitogenic signals can induce a senescence response (44,45). For example, oncogenes that deliver strong mitogenic signals cause normal cells to senesce (46–48); they do not contribute to malignant transformation unless the cells harbor mutations that allow them to ignore or bypass senescence-inducing signals (Figure 16-1; 46–48). What do all the stimuli that cause cellular senescence have in common? All of them have the ability to facilitate malignant transformation.

Cellular Senescence: Control

Consistent with cellular senescence being tumor suppressive, this process is controlled by p53 and pRB, arguably the two most potent tumor suppressor proteins encoded by mammalian genomes (Figure 16-1; 8,49–51). p53 and pRB each govern a major tumor suppressor pathway composed of several upstream regulators and downstream effectors (Figure 16-2; 52–54). Mutations in p53, pRB, or components of the pathways they govern are among the most common lesions found in cancer cells.

Some components of the p53 and pRB pathways are tumor suppressors in their own right—for example, p16, a positive regulator of pRB, and ARF, a positive regulator of p53. In addition, components of the p53 and pRB pathways suppress the activities of oncogenes and stimulators of cell proliferation. For example, ARF suppresses H/MDM2 (human/mouse double minute 2), which facilitates p53 degradation, and p16 inhibits CDKs

(cyclin-dependent protein kinases) that phosphorylate and inactivate pRB (55,56). Moreover, the p53 and pRB pathways interact (Figure 16-2). For example, pRB suppresses the activity of E2F1, a transcription factor that stimulates the expression of genes needed for DNA replication but also up-regulates the expression of ARF (57). Likewise, p53 increases the transcription of p21 (58), another CDK inhibitor that helps maintain pRB in its unphosphorylated, active form. Finally, both the p53 and pRB pathways regulate cell fates other than senescence (Figure 16-2). For example, activation of the p53 pathway can lead to cell death (apoptosis), and activation of either pathway can lead to a transient cell cycle arrest (59,60). The mechanisms by which the p53 and pRB pathways trigger one cell fate over another are incompletely understood, but both the cell type and nature of the stimulus are likely important variables.

The p53 and pRB pathways respond to different primary stimuli. Stimuli that induce a DNA damage response generally

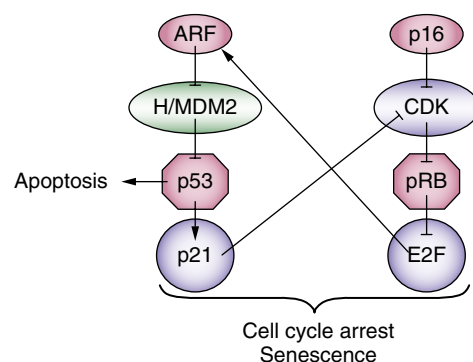


FIGURE 16-2 Cellular senescence is controlled by the p53 and pRB tumor suppressor pathways. The p53 and pRB proteins are at the hub of interacting tumor suppressor pathways that are composed of upstream regulators and downstream effectors. Within the p53 pathway, the tumor-suppressor ARF inhibits the activity of the oncogene H/MDM2, which facilitates p53 degradation, whereas p53 transcriptionally up-regulates the cyclin-dependent kinase (CDK) inhibitor p21. Within the pRB pathway, the p16 tumor suppressor inhibits CDKs, which phosphorylate and inactivate pRB, whereas pRB inhibits the activities of E2F transcription factors; E2F stimulates cell proliferation by up-regulating the transcription of growth-promoting genes, but also transcriptionally up-regulates ARF, which ultimately suppresses cell proliferation by activating p53. Both the p53 and pRB pathways can cause either a transient growth arrest (cell cycle arrest) or permanent growth arrest (senescence). Additionally, p53 activation can cause apoptosis.

activate the p53 pathway, typically by causing posttranslational modifications to p53 (61). These stimuli include dysfunctional telomeres, as well as oncogenes such as mutant RAS, which delivers a strong mitogenic signal, causing aberrant DNA replication and subsequent DNA breaks (62,63). By contrast, the pRB pathway is thought to be activated by poorly defined stresses, which typically induce p16 (54). In culture, inadequate growth conditions induce p16 (64); this induction is unlikely to be a culture artifact because p16 is also sporadically induced in vivo, and the frequency of induction increases with age (65,66). In addition, mitogenic signals and oncogenes such as mutant RAS induce p16 expression (45). Although p16 expression, in the absence of p53 activation, is sufficient to cause an irreversible senescence growth arrest (67–69), in at least some cells, stimuli such as short dysfunctional telomeres, which induce senescence by activating p53, also induce p16, albeit after a prolonged interval, and thus also activate the pRB pathway (Figure 16-2; 70–73).

Some cells spontaneously lose the ability to express p16, often due to promoter methylation. This is true in culture (69,74) and in vivo (75,76). Such cells remain susceptible to p53-mediated senescence. For example, cells that cannot express p16 still senesce after repeated cell division owing to critical telomere shortening and activation of the p53 pathway. Notably, however, inactivation of p53 in cells that have senesced without p16 expression causes them to reenter the cell cycle and proliferate—often with dysfunctional telomeres or damaged DNA (69,77). Consequently, such cells are at increased risk for malignant transformation. By contrast, once cells senesce with elevated p16 expression, the senescence growth arrest cannot be reversed by subsequent inactivation of p53, p16, or pRB (69). Thus, the senescence arrest mediated by the p16/pRB pathway provides a formidable second barrier to the proliferation of potentially tumorigenic cells with damaged DNA.

Mouse–Human Differences

Mouse models in which cells are defective in undergoing a senescence response support the idea that cellular senescence suppresses tumorigenesis in vivo. However, although mice are valuable models for human disease, there are some striking mouse–human differences that need to be considered in interpreting data from mouse models. In contrast to human cells, in which telomeres typically range from 10 to 15 kb, cells from laboratory mice have long, heterogeneous telomeres (up to >50 kb); moreover, mouse tissues and cultured cells often express telomerase (78,79). Thus, mouse cells generally do not undergo senescence due to telomere shortening, although they do undergo senescence in response to DNA damage, oncogenes, and other stimuli (80,81). Nonetheless, mouse cells also have only a limited proliferative capacity in culture. This decline in proliferation is due to the severe oxidative damage caused by the hyperphysiologic oxygen concentrations used in standard culture conditions (82). Thus, the proliferation of mouse and human cells differs significantly in culture, owing to differences in telomere biology and susceptibility to oxidative stress. With this caveat in mind, mouse models have nonetheless provided important information regarding the role of cellular senescence in suppressing the development of cancer.

Cellular Senescence Suppresses Tumorigenesis In Vivo

Cell culture experiments have unambiguously established the importance of the p53 and p16/pRB pathways for the senescence response of human and mouse cells (51,56,83). Moreover, human and mouse tumors inevitably harbor mutations in genes encoding components of these pathways, suggesting that cellular senescence plays an important role in suppressing cancer. Mouse models, and recent studies of premalignant and malignant tumors from humans, have provided some of the strongest evidence for this idea. Some of these studies use characteristic markers, none of which are exclusive, to identify senescent cells in tissues. These markers include expression of a neutral β -galactosidase (84), expression of p16 (65), and distinct nuclear structures (foci) of heterochromatin or DNA damage proteins (85–87).

Among the earliest mouse models that were defective in mounting a senescence response, were mice with germ-line deficiencies in the genes encoding p53 or p16. These mice develop normally but are cancer-prone. The p53-deficient mice develop malignant tumors within about 6 months of age (88). In addition, cells from these mice are highly resistant to the growth arrest seen under standard culture conditions, as well as senescence induced by DNA damage and oncogenes such as RAS (89). However, p53-deficient mouse cells are defective in both apoptosis and senescence (89), so the extreme cancer susceptibility of these mice is likely due to the combined defects in apoptosis and senescence. The p16-deficient mice, by contrast, develop tumors later in life—after 12 to 18 months of age, which is still in advance of the average age at which wild-type mice develop cancer (18–24 months, depending on the mouse strain; 90). Cells from these mice do not appear to be defective in apoptosis, and retain, to a large extent, the ability to arrest growth under standard culture conditions and senesce in response to oncogenic RAS. Thus, p16-deficient cells retain the ability to senesce in response to stimuli that activate the p53 pathway, but are defective in pRB-mediated senescence. Since these mice are cancer-prone, these observations suggest that p16-mediated senescence suppresses malignant tumorigenesis in vivo.

Recent findings substantiate a role for cellular senescence in suppressing the development of cancer in mice and humans. In two mouse models, germ-line engineering was used to express oncogenic RAS proteins in several mouse tissues (91,92). In one case, senescent cells were found in premalignant lesions of the lung, skin, and pancreas, but not in the malignant tumors that developed in these tissues after a long latency (92). Likewise, oncogenic RAS induced senescence when expressed in lymphocytes, but lymphomas formed only in mice that were additionally engineered to be deficient in Suv39h1, a histone methyltransferase that acts in the pRB pathway and mediates cellular senescence (91). In a third mouse model (93), prostate-specific inactivation of the PTEN tumor suppressor, which encodes a phosphatase that dampens mitogenic signals, led to the appearance of premalignant prostatic lesions containing numerous senescent cells. When these mice were crossed to p53-deficient mice, senescent cells were not evident and lethal invasive cancers developed. Moreover, senescent cells were found in early-stage prostate cancer in humans, but not

in highly malignant late-stage cancers (93). Similarly, oncogenic mutations in BRAF, a component of the RAS signaling pathway, elicit a senescence response in human fibroblasts and melanocytes in culture. In human skin biopsies, senescent cells are found in benign melanocytic nevi, but not in malignant melanomas (48).

Taken together, these studies strongly suggest that oncogenic mutations that result in strong mitogenic signals cause cellular senescence *in vivo*, and that the senescence response is important for suppressing the progression of premalignant lesions to lethal malignant tumors. Moreover, malignant progression occurs when cells acquire additional mutations, primarily in the p53 or pRB pathways, which allow them to either ignore senescence-inducing signals or escape from the senescence growth arrest.

Cancer and Aging

Cancer is an age-related disease in mammals with the vast majority of malignant tumors occurring after about the midpoint of the species-specific life span (approximately 1.5 years in mice and 50 years in humans; 94,95). This age-dependence may not simply reflect the time needed to acquire the requisite number of somatic mutations. First, mutations, including oncogenic mutations, begin to accumulate very early in life and are present in apparently normal tissues (Figure 16-3; 96–98). Second, the development of cancer from cells with oncogenic mutations depends to a large extent on the tissue microenvironment. Normal tissue structures and microenvironments can suppress, and in some cases even reverse, malignant tumorigenesis (99–102). Thus, age-related cancers may arise owing to at least two synergistic processes—an accumulation of mutations and a decline in normal tissue structure and function,

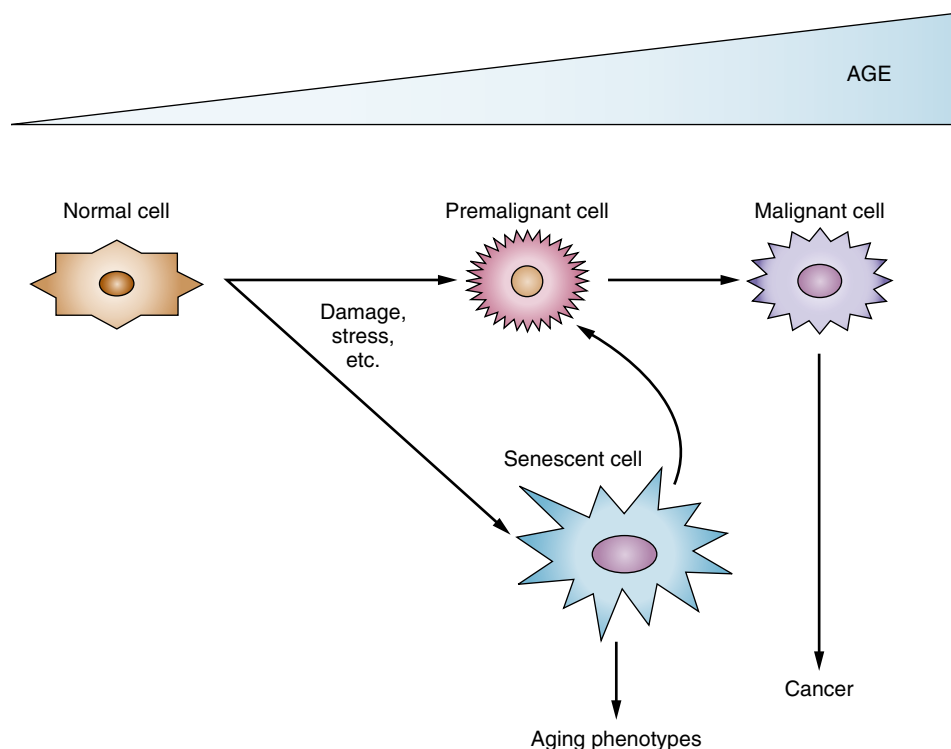
both of which increase with age (Figure 16-3; 21,95). Mutation load most likely increases with age owing to DNA damage from exogenous and endogenous sources and errors in DNA replication. In addition, DNA replication and repair can cause epimutations—mistakes in the modifications to DNA, histones, and other chromatin-associated proteins that determine patterns of gene expression, which are also important causative factors the development of cancer (103,104). The age-related decline in tissue integrity most likely has many etiologies, one of which has been proposed to be the accumulation of senescent cells (Figure 16-3).

Cellular Senescence and Aging

Senescent cells have been shown to increase in number with age in a variety of renewable mammalian tissues (84,86,105–107). The etiology of these senescent cells is difficult to reconstruct but, in at least some cases, their accumulation can be attributed to an increase in the number of cells with dysfunctional telomeres (85,86). In addition, senescent cells have been found at sites of age-related pathologies, especially degenerative pathologies such as osteoarthritis, intervertebral disc degeneration, venous ulcers, and atherosclerosis (107–112). At face value, these findings support the idea that cellular senescence recapitulates some aspects of organismal aging, as initially proposed by Hayflick and colleagues. However, these data also suggest that the age-dependent increase in senescent cells might actively contribute to phenotypes and diseases associated with aging.

Why might senescent cells contribute to aging? As noted earlier, a common feature of senescent cells is increased expression of genes encoding secreted molecules (15–20). These molecules

FIGURE 16-3 Senescent cell may fuel both aging phenotypes and cancer. During aging, cells experience damage and a variety of stresses that can induce cellular senescence or, in some cases, cause mutations in genes required for the senescence response, giving rise to a premalignant cell. Senescent cells secrete molecules that can compromise tissue integrity, thereby promoting phenotypes associated with aging. Senescent cells can also create a procarcinogenic tissue microenvironment, which can then promote the progression of premalignant cells to full-blown malignancy.



include inflammatory cytokines, chemokines, growth factors, and proteases—molecules that can radically alter the local tissue microenvironment. Thus, senescent cells might contribute to the age-related decline in tissue structure and function that is a hallmark of aging organisms (Figure 16-3; 113). The evidence for this hypothesis is indirect. Aside from the presence of senescent cells in aging and degenerating tissues, heterotypic cell culture models show that the presence of senescent cells can, at least in principle, disrupt the normal morphologic and functional differentiation of mammary and skin epithelial cells and microvascular endothelial cells (114–117).

Cellular Senescence and Antagonistic Pleiotropy

Why might cellular senescence, an established tumor-suppressive mechanism, contribute to aging? This question might be framed in an even larger context. How can any fundamental process be both beneficial (tumor suppressive) and detrimental (pro-aging)?

As noted previously, cancer poses a major challenge to the longevity of organisms with renewable tissues. This challenge arises from the fact that cell proliferation is essential for regeneration and repair, and hence is essential for the health of organisms with renewable tissues, but is also essential for tumorigenesis (7). Moreover, the mitotic cells that comprise renewable tissues are more prone than postmitotic cells to acquiring mutations (118), a major cause of cancer. Of course, the risk posed by cancer has been mitigated by the evolution of tumor-suppressor mechanisms such as cellular senescence. However, the evolution of at least some tumor-suppressor mechanisms, cellular senescence in particular, may have entailed an evolutionary trade-off (3).

In general, organisms evolved in environments that were replete with extrinsic hazards, including predation, starvation, and infection. Thus, throughout most of the evolutionary history of most organisms, life span was limited by death due to external catastrophes. Consequently, survival mechanisms such as tumor suppression needed to be effective only up to and during the period of peak reproduction, a few decades for humans and several months for mice. Should a tumor-suppressive mechanism have deleterious effects after the peak reproductive age, there would be little selective pressure to eliminate them. Thus, it is at least theoretically possible for a tumor suppressive mechanism to be both beneficial and detrimental, depending on the age of the organism. This idea—that a biologic process can benefit organisms early in life but have *unselected* effects that have escaped the force of natural selection and are deleterious later in life—comprises an important evolutionary theory of aging termed “antagonistic pleiotropy” (119,120).

Cellular senescence might be an example of evolutionary antagonistic pleiotropy. As such, the selected phenotype was the arrest of cell proliferation, whereas the changes in gene expression, particularly those that result in the secretion of molecules that can alter tissue microenvironments, might have escaped the force of natural selection. The origin of the senescent phenotype might be the tissue-wounding response because, at least for stromal fibroblasts and endothelial cells, the secretory phenotype of

senescent cells resembles the response to a wound (113,121). Following wounding, there is a transient burst of cell proliferation, but this is followed by an arrest of proliferation and the secretion of molecules that can attract immune cells, remodel the extracellular matrix, and stimulate the growth of neighboring cells, such as adjacent epithelial cells. Senescent cells might be frozen in the post-replicative, but activated, secretory stage. Although the presence of such chronically activated cells would not necessarily be beneficial—such cells would create local sites of chronic inflammation, tissue degradation and fibrosis, and hyperproliferation—they may not affect tissue homeostasis early in life, when their numbers are low, but become deleterious only later in life, when their numbers increase (Figure 16-3).

Consistent with this idea, mouse models in which p53 is chronically activated—in these cases by expression of artificially generated or naturally occurring short p53 isoforms—confer extraordinary protection against cancer, but accelerate the development of multiple aging phenotypes (122,123). Cells from these mice are prone to both apoptosis and cellular senescence (122,124). In addition, cells that spontaneously express p16, which were likely but not formally demonstrated to be senescent, increased with age in the stem or progenitor cell compartments of the subventricular zone of the brain, hematopoietic system, and pancreatic islets of mice. Concomitantly, mice show a decrease in neurogenesis and ability of the bone marrow to reconstitute an immune system and an increase in diabetes. These age-related disorders were retarded in p16-deficient mice, despite their predisposition to developing cancer (125–127).

Cellular Senescence and Cancer

As noted previously, cancer incidence increases with age and may be fueled by the dual processes of mutation accumulation and age-dependent changes in tissue integrity. Moreover, the presence of senescent cells can, at least in principle, contribute to the tissue changes that occur during aging. Further, the secretory phenotype of senescent fibroblasts resembles the phenotype of carcinoma- or tumor-associated fibroblasts, stromal cells that are activated by carcinomas and produce extracellular factors that promote epithelial carcinogenesis (128,129). These findings predict that senescent cells may also, ironically, promote the development of cancer late in life. In cell culture and mouse xenograft experiments, senescent cells stimulate the ability of premalignant or malignant epithelial cells to both proliferate and invade a basement membrane (18,116,130–132), two essential steps in the development of cancer. In addition, senescent cells produce angiogenic factors such as VEGF (vascular endothelial growth factor), which can stimulate normal endothelial cells to invade a basement membrane in culture, and may be responsible for the increased vascularity of xenografted tumors that form in the presence of senescent fibroblasts (133). Together, these findings support the idea that cellular senescence, despite being a potent tumor suppressive mechanism, is antagonistically pleiotropic and contributes to age-related pathology, including late-life cancer (Figure 16-3).

Unanswered Questions and Future Directions

More than 40 years after the seminal findings of Hayflick and colleagues were first reported, the idea that cellular senescence suppresses the development of cancer has gained substantial support. In this regard, the senescence response resembles apoptosis, the other tumor-suppressive mechanism that acts at the level of cell fate.

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The Tumor Microenvironment in Cancer Progression

Over 100 years ago, Paget hypothesized that the “soil” was as important to the development of tumors as the tumor “seed” itself (reviewed in [1]). Nevertheless, until recently, the study of cancer has concentrated on the genetic and molecular description of cancer cells, which are predominantly epithelial in origin, or the laboratory techniques used to study these cells as they become abnormal during tumor progression. After years of research focused almost exclusively on oncogenes and tumor suppressors, the study of cells that surround and respond to the neoplastic epithelium in the tumor microenvironment or tumor stroma is expanding at a remarkable rate. It is becoming clear that the microenvironment acts as a coconspirator during carcinogenesis and neoplastic progression. Although there has been significant interest in the vascular response to tumors, so-called angiogenesis, other components have been scarcely studied until recently. This remarkable change in research focus occurred as scientists realized that studying cancer without studying the tumor microenvironment would give an incomplete story of the factors that contribute to cancer progression, like listening to a symphony with only one musician performing, and would ignore many of the cells often seen in careful analysis of carcinomas.

In this chapter, we focus on defining the role of the microenvironment during tumor progression and on describing several of the better characterized tumorigenic stromal components, including macrophages, fibroblasts, and some of the molecules involved in the communication between the microenvironment and the cancer cells, including fibroblast-secreted protein-1 (FSP-1, mts-1/metastasin/S100A4), transforming growth factor- β (TGF- β), the chemokine CXCL-12 (stromal-derived factor-1 α , SDF-1 α), type I collagen, matrix metalloproteinase (MMP)-13, MMP-3, and MMP-14 (membrane-type MMP, MT1-MMP).

Tumor Microenvironment: the Coconspirator of Cancer Progression

Histologic examination of tumors shows that many nonepithelial cell types are present in tumors, comprising the tumor stroma. The stromal microenvironment consists of fibroblasts, adipocytes, macrophages, mast cells, vascular components, and inflammatory cells

of the innate and acquired immune system, as well as the extracellular matrix (ECM) and all the molecules that are concentrated and immobilized on it. All of these components communicate with each other and with the neoplastic cells to contribute to the aberrant tumor organ, and it is generally accepted that the stromal microenvironment contributes to tumorigenesis in cancers of epithelial origin. Although the epithelium in the carcinoma certainly is mutated, the events that promote tumor progression involve the stroma (Figure 17-1). In fact, in some cases, the trigger for neoplastic progression may even come from signals within the stromal microenvironment (reviewed in [2,3]).

The microenvironment has such a powerful impact on the status of the cells that a normal microenvironment can even prevent malignant cells from committing to neoplasias. In classic experiments, Beatrice Mintz in the 1970s showed that mouse teratoma embryonic carcinoma cells passaged for 8 years as ascites tumors can revert to cells comprising normal adult tissue if injected into the normal environment of blastocysts from teratoma-free mice (4). In this context, these cells, which were malignant under the regulation of another microenvironment, can instead be induced to differentiate into the normal tissues of the mouse and do not form tumors (Figure 17-2). The stunning conclusion is that restoration of normal microenvironmental signaling can reverse the malignant phenotype even though the cancer cells retain all their mutations (5,6).

Although normal stroma may protect the epithelium from tumorigenesis, aberrant stroma can initiate tumorigenesis (7–11). These stromal cells appear to carry on many normal functions, but they drive transformation by hijacking normal cellular responses and inducing them at the wrong place or at the wrong time. In most cases the stroma is “activated,” or tumor promoting, but genotypically normal; however, tumor-suppressor gene mutations within the stroma can be found (12).

A classic example of a stromal signal that can trigger neoplasms is chronic *inflammation*. As early as 1863, Rudolf Virchow saw a connection between inflammation and cancer when he saw leukocytes infiltrating early neoplasias, which were caused by chronic inflammation (13). Epidemiologic evidence supporting an association of inflammation with cancer comes from studies showing a relationship between inflammatory bowel disease and colon

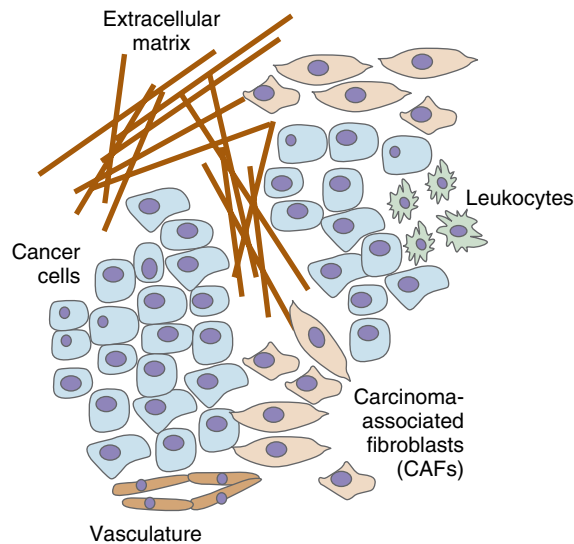


FIGURE 17-1 THE TUMOR MICROENVIRONMENT. Surrounding the epithelial cancer cells (blue) are the components of the tumor microenvironment, which includes the extracellular matrix (ECM; orange), the carcinoma-associated fibroblasts (CAFs; peach), leukocytes (green), and vasculature (red). (From Egeblad M, Littlepage LE, Werb Z. The fibroblastic coconspirator in cancer progression. *Cold Spring Harb Symp Quant Biol* 2005;70:383–388, with permission.)

cancer, between *Helicobacter pylori* infection of the stomach and stomach cancer, and between hepatitis C infection and hepatocellular carcinoma (reviewed in [14]). Further experimental evidence for the link between inflammation and stromal promotion of cancer comes from the studies on two-stage carcinogenesis, in which mutagens alone do not produce tumors but instead require the

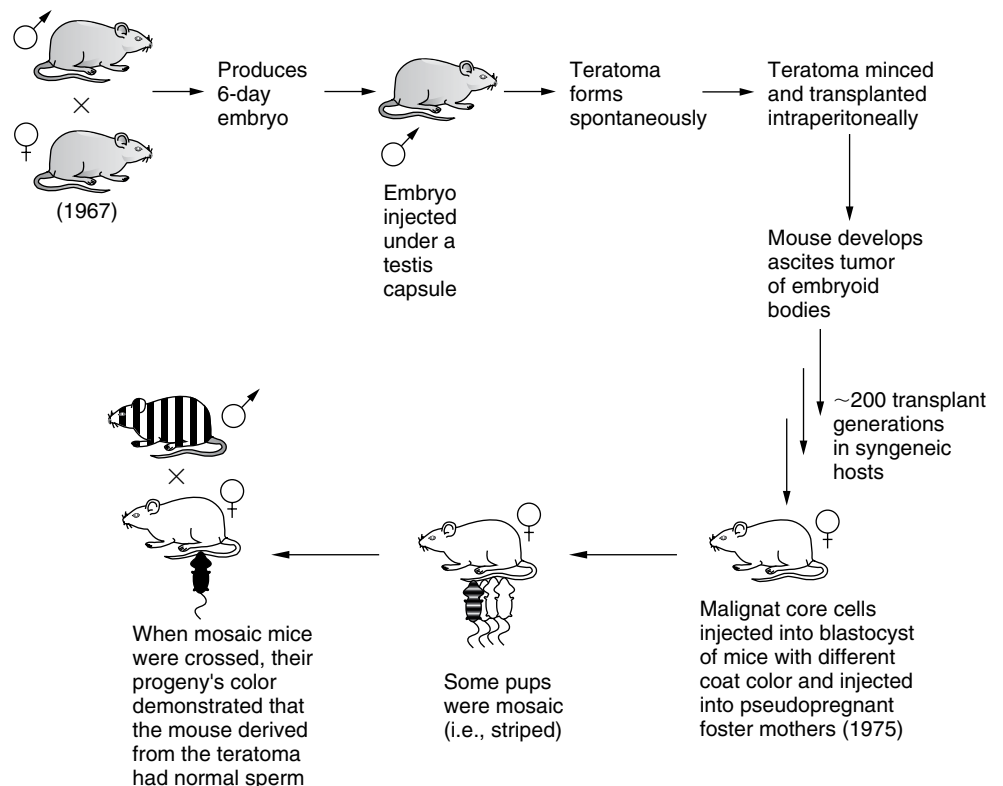
application of *tumor promoters*, such as *phorbol esters*, which can occur long after carcinogen exposure. The tumor promoters trigger an inflammatory response and generate an aberrant tumor-promoting stroma. Another process that can generate tumor-promoting stroma experimentally is *irradiation*. Irradiation of the mammary gland induces nonreversible changes in the stroma that contribute to neoplasia: Nontransformed mammary epithelial cells injected into irradiated mammary stromal fat pads have greatly increased tumor growth compared with those injected into the contralateral, nonirradiated mammary fat pads (15). Similar results have been obtained when comparing nonirradiated fibroblasts with irradiated fibroblasts, where only the latter stimulates invasiveness of pancreatic cancer (9).

Macrophages Promote Tumor Progression

Macrophages, which are hematopoietic cells in the innate immune system, function to phagocytose cellular debris and foreign particles (including aberrant cancer cells) and present antigens to T cells during normal immune responses. They aid in the immune response and tissue repair by secreting factors that recruit more macrophages and additional immune cells to the wound site. Macrophages are influenced by their microenvironment during their normal function and are differently activated, depending on the tissue and the microenvironment.

Even though macrophages in a normal tissue should be responsible for removing tumorigenic cells, much evidence points to macrophages as being active coconspirators in cancer progression

FIGURE 17-2 MALIGNANT EMBRYONIC CARCINOMA CELLS CAN FORM NORMAL TISSUE IF THEY ARE IN THE RIGHT CELLULAR MICROENVIRONMENT. Mouse teratoma embryonic carcinoma cells were passaged for 8 years as ascites tumors and injected into the normal environment of blastocysts from mice free of tumors. Under these conditions, given the right microenvironment, the carcinoma cells were able to form normal adult tissues. (From Mintz B, Illmensee K. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc Natl Acad Sci U S A* 1975;72:3585–3589, with permission.)



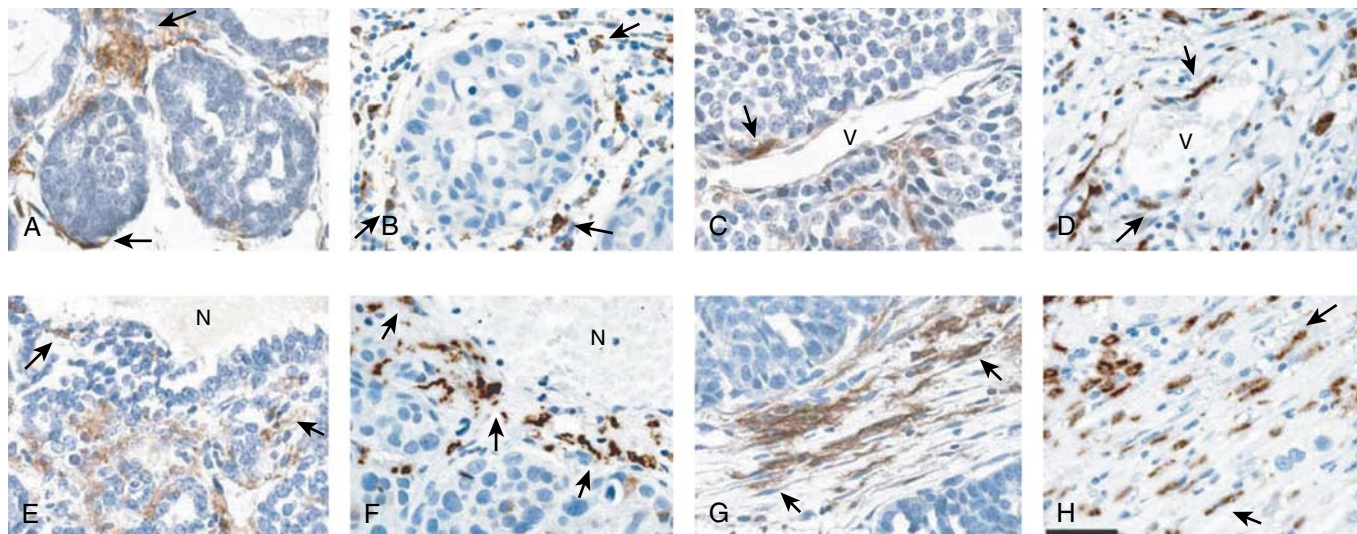


FIGURE 17-3 MACROPHAGES ARE FOUND IN DIFFERENT AREAS OF TUMORS. Tumor-associated macrophages (TAMs) seen here are visualized from fixed sections of tumors from polyoma middle T (PyMT)-induced tumors in mouse mammary glands (**A, C, E, G**) or human breast carcinomas (**B, D, F, H**) using antibodies that specifically recognize the pan-macrophage markers F4/80 (murine) or CD68 (human). TAMs are seen to gather in areas of cancer cell invasion where they likely help to degrade the basement membrane and facilitate the migration of cancer cells into the stroma of the surrounding tissue (**A, B**). They are also found in perivascular areas where they have been proposed to promote metastasis by expressing factors such as endothelial growth factor (**C, D**). Other subpopulations of TAMs are found in hypoxic, perinecrotic areas (**E, F**) where they likely promote angiogenesis and metastasis. Finally, TAMs are also found in purely stromal areas (**G, H**). Bar, 50 μ m; V, blood vessel; N, necrosis. (From Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 2006;66:605–612, with permission.)

through responses to microenvironmental changes that modify macrophage abilities and functions. Most studies have found that a high density of *tumor-associated macrophages* (TAMs) correlates with poor prognosis and reduced survival in a number of different cancers (e.g., breast, prostate, endometrial, bladder, kidney, esophageal, squamous cell carcinoma, malignant uveal melanoma, follicular lymphoma; reviewed in [16–19]; Figure 17-3).

Macrophages, in particular, play a dual role stimulating angiogenesis and tumor growth [14,16–18,20,21]. Although macrophages are the dominant leukocyte population [16,22], neutrophils are also factors in tumor progression [23]. Considerable evidence indicates that macrophages regulate tumor angiogenesis, in part by producing vascular endothelial growth factor (VEGF; [19]) and then mobilizing it through the production of proteolytic enzymes [24,25]. Finally, macrophages and related myeloid cells may prepare a niche in the distant sites that facilitate the metastatic growth of the disseminated cells [26].

Genetic studies using mouse models demonstrate an important role for macrophages in carcinogenesis. Colony-stimulating factor-1 (CSF-1, M-CSF), a monocyte/macrophage lineage growth factor, is one example of a molecule that plays a role in mouse models of cancer. One genetic study used a CSF-1 null animal (Csf1^{op/op}) and crossed it with a mammary tumor progression model overexpressing polyoma middle T (PyMT) to investigate the role of macrophages in neoplastic development. Consistent with Csf-1 playing a role in macrophage function, Csf1^{op/op}; PyMT mice with null expression of Csf-1 have reduced tumor infiltration of macrophages compared with the PyMT control mice. Neoplastic progression and proliferation in the primary tumor remain unaffected by the loss of Csf-1; however, metastasis to lung is dramatically delayed in the absence of Csf-1 [27].

Tumor-associated macrophages are recruited to the tumors through cytokines and chemokines secreted by the cancer cells [19,22,28]. Unlike macrophages in a normal, healthy tissue or wound-healing environment, TAMs are modified in the context of the tumor microenvironment and lose the ability to phagocytose cancer cells or present tumor antigens to T cell [18]. Through the secretion of factors, including growth factors and proteases, macrophages promote cancer cell proliferation, survival, motility, and growth. Thus, the macrophages contribute to tumor progression at several stages, from the chronic inflammatory response and tumor initiation, matrix remodeling, angiogenesis, tumor invasion, and intravasation to metastasis.

Macrophages Act at Sites of Chronic Inflammation and Promote Tumor Progression

As one of the key players in inflammatory response, macrophages are at the site of chronic inflammation where they recruit other cell types. They actually create a mutagenic environment through the secretion of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are known to cause lesions in DNA, RNA, proteins, and membranes through their free-radical intermediates, and these defects can drive carcinogenesis [29]. In fact, people with chronic inflammatory disease are prone to certain types of cancer [29].

Landmark studies suggest that macrophages promote both early and late events during tumor progression. Macrophages can be found at several different locations in tumors probably due to different functions in tumor progression. First, they are found infiltrating the tumor at sites undergoing basement membrane breakdown, which is necessary for cancer cell invasion into

the surrounding stroma (27). In addition, macrophages release cytokines and chemokines that promote invasiveness of the cancer cells. Coculturing cancer cells with macrophages increases invasiveness, a process that is dependent on $\text{TNF-}\alpha$ and matrix metalloproteinases (30). Macrophages are also found in hypoxic areas in tumors (18). Their presence in these areas may lead to up-regulation of secreted angiogenesis stimulating factors such as VEGF (22), one of the proposed mechanism by which TAMs may promote angiogenesis (17,31). Finally, macrophages and related myeloid cells encourage metastasis and seeding at distant sites by facilitating the dissemination of cancer cells from the primary site (16) and nurturing the cancer cells to establish at the secondary site (26). Imaging of tumors by intravital confocal microscopy supports the suggested role of macrophages promoting invasion and intravasation of the blood vessels. These images have captured macrophages in action, both surrounding blood vessels and interacting with the tumor (16,32).

Fibroblasts Influence Tumor Progression

The histology and expression profiling of the tumor microenvironment shows similarities between invasion and metastasis to a normal wound-healing response, causing some scientists to suggest that the tumor microenvironment is “normal wound healing gone awry” (33). Fibroblasts, one of the cellular components of the stroma, are derived from mesenchymal cells and adapt to tissue injury. During wound healing, they change their phenotype to become “reactive.” Although fibroblasts taken from different anatomic sites have their own expression profiles that are dependent on their position and differentiation state, tumor-associated fibroblasts mimic a wound response, creating a reactive stroma that promote carcinogenesis (34–36). Examination of the expression profiles of fibroblasts taken from ten different anatomic sites and exposed to serum gives a fibroblast serum-response expression program, with a similar signature seen in the tumor (34–36).

The reactive fibroblast is also known as a myofibroblast—a cell type that shares properties with fibroblasts and the smooth muscle cells. The reactive fibroblasts that arise during neoplasia are often referred to as carcinoma-associated fibroblasts (CAFs). CAFs differ from normal fibroblasts by having an abnormally high expression of smooth muscle actin and increased expression of proteolytic enzymes and ECM proteins, such as tenascin-C.

CAFs contribute directly to epithelial carcinogenesis, as has been established in recombination experiments. For example, when immortalized, nontumorigenic human prostate epithelial cells are mixed with CAFs from human prostate carcinomas and grafted under the kidney capsules of immune-deficient mice, the epithelial cells develop into large carcinomas. In contrast, mixing the immortalized epithelial cells with fibroblasts from a normal prostate gland does not result in carcinoma formation (10).

What is a Carcinoma-Associated Fibroblast?

Although we are starting to understand what molecules are secreted by CAFs and what their functions are, we know surprisingly little

about what the tumor-promoting CAFs themselves are and what distinguishes them from the normal fibroblasts. Like true fibroblasts, CAFs express the intermediate filament vimentin. However, they also express α -smooth muscle actin and can contract collagen gels in vitro, thereby resembling myofibroblasts. Thus, the origin of these cells is unclear (Figure 17-4). They could be derived from fibroblasts or fibroblast precursors and change into CAFs through stimulation by the carcinoma cells. Indeed, it has been shown that when normal fibroblast are cocultured with carcinoma cells, the fibroblast undergo a myofibroblastic conversion (37).

Interestingly, the CAFs maintain their ability to stimulate tumor progression through several cell passages, but show no evidence of genetic alterations and senesce normally in culture (38). Thus, this would suggest a nonreversible epigenetic change of the fibroblasts. However, the CAFs ability to stimulate carcinogenesis, even after having been cultured for several generations without further stimulation from the cancer cells, make it tempting to speculate that the CAFs are an expanded population of an early developmental precursor initially present in the normal precancerous tissue, a population that first expands in response to signals from the cancer cells. Indeed, cells with CAF-like properties are present before tumors evolve: when fibroblasts are isolated from breast tissue from patients undergoing surgery for either benign mammary gland lesions or cancer, the cancer patients' fibroblasts are more motile than the fibroblasts from the patient with benign lesions, even though the fibroblasts are isolated in normal tissue, away from the cancer or the benign lesion (39). The enhanced motility of cancer patients' fibroblasts is also found in fetal fibroblasts and is due to the secretion of an oncofetal migration stimulating factor that is an alternatively spliced, truncated form of fibronectin (40). These data therefore suggest that the presence of fibroblasts with fetal or CAF-like properties predisposes for the development of cancer.

Can germ-line mutations also determine fibroblast behavior? The premalignant hamartomatous lesions of juvenile polyposis coli, which arise from germ-line mutations in the *SMAD4* gene, are largely fibroblastic in nature, and thus a mutation in the stromal compartment initiates the development of the premalignant lesions that eventually lead to colon cancer (41–43). Thus, the stroma also can be the target of somatic mutations and, at least in some cases, the mutations found in the fibroblastic cells and the carcinoma cells are different (12,44,45). This strongly suggests that the CAFs are not derived from the carcinoma cells (by for example, epithelial-to mesenchymal transition [EMT]), but rather that the mutations have arisen independently in the two cell populations.

However, CAF-like cells may be derived from carcinoma cells that have undergone EMT. Indeed, immortal fibroblast-like cells have been isolated from human breast cancer that had the same X-inactivation pattern as the epithelial carcinoma cells in the tumor (46). The cells are not tumorigenic by themselves, but behaved like CAFs, stimulating epithelial carcinoma cells activation of MMPs in vitro and tumor growth in vivo (46). The chondrous metaplasia seen in some breast and ovarian cancers arise from cancer cells that have undergone EMT and then mesenchymal differentiation to cartilage (7).

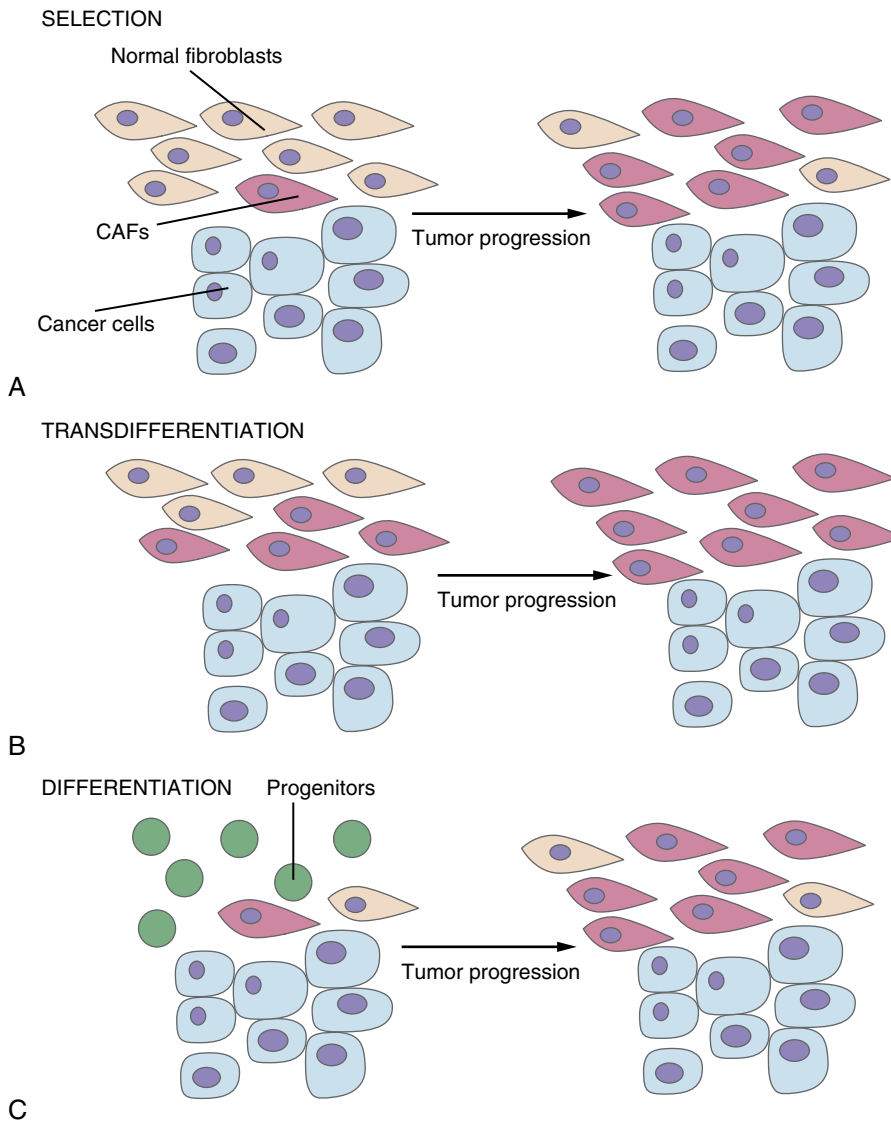


FIGURE 17-4 POSSIBLE MODELS FOR GENERATION OF CARCINOMA-ASSOCIATED FIBROBLASTS WITHIN CARCINOMAS. **A:** Clonal selection from a small population of fibroblasts or progenitors that have undergone genetic alterations. **B:** Transdifferentiation from normal cells, such as normal fibroblasts. **C:** Differentiation from progenitor cells. (From Orimo A, Weinberg RA. *Stromal fibroblasts in cancer: a novel tumor-promoting cell type.* *Cell Cycle* 2006;5:1597–1601, with permission.)

Thus, it appears that tumors have developed multiple different ways to ensure that CAF-like cells are present in the tumor organ: CAFs may be an expanded precursor mesenchymal cell population, epigenetically changed fibroblasts, mutated fibroblasts, or even epithelial cells that have undergone EMT. Why have the tumors developed so many different ways of recruiting CAFs? Do the CAFs arise as a part of a defense mechanism against the tumor—an attempt to encapsulate the tumors—or do the cancer cells recruit the CAFs (by either of the mechanisms mentioned above) because the communications between the cancer cells and the CAFs are crucial for the tumor progression? These are some of the important questions to be addressed in the future.

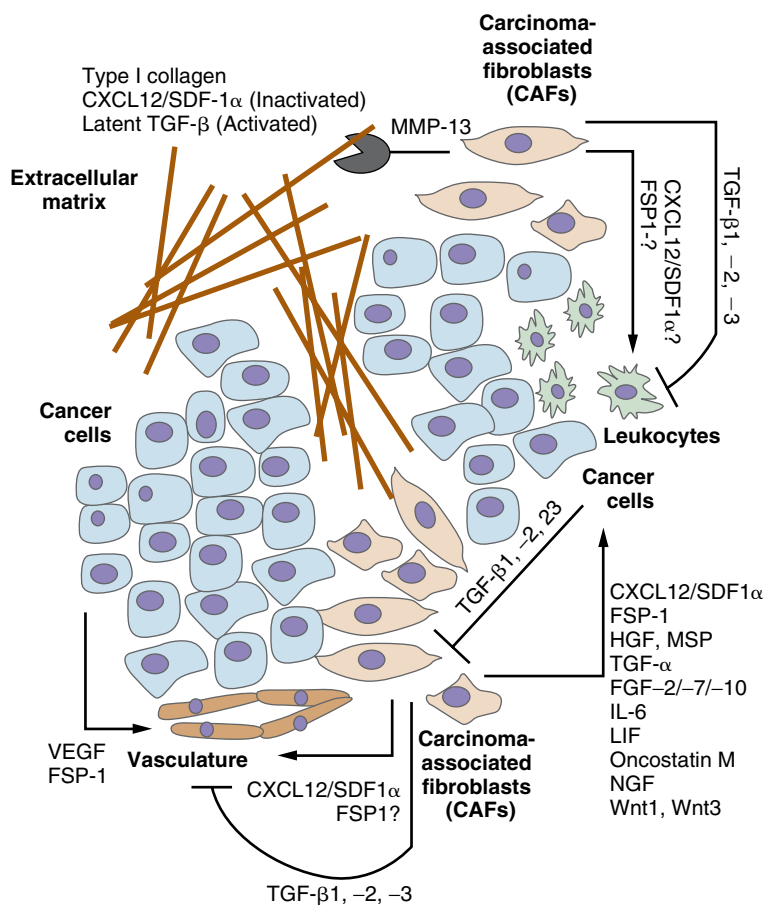
Fibroblasts Stimulate Epithelial Cancer Progression Through Secreted Factors

Although recombination experiments illustrated the effects of CAFs on epithelial carcinogenesis, only some of the molecules responsible for these effects have been identified. However, most

of these molecules are not exclusively expressed by the CAFs in the carcinomas. An example is fibroblast secreted protein (FSP1, also called S100A4, metastasin or mts1), which is expressed in CAFs, carcinoma cells, and macrophages during tumor progression (47,48). FSP1 is a calcium-binding protein with intracellular and extracellular protein-binding partners. Intracellularly, it interacts with and possibly inactivates p53. FSP1 also interacts with nonmuscle myosin heavy chain, actin filaments, and nonmuscle tropomyosin, thereby potentially influencing the cytoskeleton and regulating cell motility (reviewed in [49]). The extracellular binding partners of FSP1 are largely unknown, with the exception of annexin II. FSP1 binds to this coreceptor for the serine proteinase plasminogen, which results in increased activation of plasminogen (50). FSP1 is proangiogenic, and this may be mediated by plasminogen activation or up-regulation of matrix metalloproteinase (MMP) 13 (51), which may facilitate endothelial cell invasion (Figure 17-5).

Compelling evidence exists for FSP1 as a crucial stromal factor regulating metastasis: carcinoma cells that are metastatic

FIGURE 17-5 MOLECULAR COCONSPIRATORS OF STROMAL–EPITHELIAL INTERACTIONS DURING TUMORIGENESIS. The cells and cofactors surrounding the cancer cells communicate during tumor progression, leading to the secretion of growth factors, chemokines, and cytokines. Shown here are examples of stimulators of tumorigenesis that are secreted by one cell type and act on another through activation (arrows), inactivation (blocked lines), or proteolytic cleavage (chewing symbol). VEGF, vascular endothelial growth factor; CXCL12/SDF-1 α , stromal derived factor 1 α ; FSP-1, fibroblast specific protein-1; HGF, hepatocyte growth factor; MMP-13, matrix metalloproteinase 13; MSP, macrophage-stimulating protein; TGF- α ? transforming growth factor- α ; TGF- β , transforming growth factor- β ; FGF, fibroblast growth factor; IL-6, interleukin 6; LIF, leukemia inhibitory factor; NGF, nerve growth factor. (From Egeblad M, Littlepage LE, Werb Z. *The fibroblastic coconspirator in cancer progression*. Cold Spring Harb Symp Quant Biol 2005;70:383–388, with permission.)



when injected into wild-type mice are less likely to form tumors and do not metastasize at all when injected into *Fsp1*^{-/-} mice. Coinjection of *Fsp1*^{+/+} fibroblasts with the cancer cells restores tumor development and metastasis in the *Fsp1*^{-/-} animals, whereas coinjection with *Fsp1*^{-/-} fibroblasts does not (52). This suggests that FSP1, when secreted by the fibroblasts, alters the stromal microenvironment, making it more favorable for tumor progression. This could be through the regulation of angiogenesis and inflammation: Tumors forming after coinjection of carcinoma cells with *Fsp1*^{-/-} cells have decreased numbers of infiltrating macrophages, smooth-muscle, actin-expressing myofibroblasts and CD31⁺ endothelial cells compared with tumors developing after coinjection with *Fsp1*^{+/+} cells (52).

FSP1 is also up-regulated in metastatic carcinoma cells, perhaps as a result of EMT, which gives the carcinoma cells a fibroblastic phenotype. EMT has been proposed to be the mechanism responsible for the metastatic phenotype induced by FSP1 (53). If FSP1 mainly exerts its tumor-promoting function as a secreted protein, the cellular source of its secretion might not be important. It is possible that FSP1 can be an important factor that induce angiogenesis, inflammation, and EMT depending on which cell type it acts on, rather than which cell type secretes it.

CXCL12 Stimulates Epithelial Carcinogenesis

The CXC chemokine CXCL12 (also known as stromal cell-derived factor-1 α , SDF-1 α) is another important factor secreted

by CAFs (38). CXCL12 acts through several mechanisms: It acts directly on the mammary carcinoma cells stimulating proliferation through the CXCL12 receptor CXCR4. CXCL12 may stimulate metastasis to the lung and to lymph nodes, which have a high expression of the chemokine, resulting in homing of the cancer cells, which express the CXCL12 receptor, to these organs (54). In addition to direct actions on the cancer cells, CXCL12 secretion by CAFs leads to recruitment of endothelial cell precursors to the growing tumor, thereby promoting angiogenesis (Figure 17-5). As mentioned previously, there is a strong link between stromal changes, inflammation, and carcinoma progression also when it comes to the effects mediated by the fibroblasts. The functions of CAF-secreted CXCL12 have only been studied using xenograft models in immune-compromised mice. However, CXCL12 is a well-established chemoattractant for leukocytes, and it is thus likely that CXCL12 has additional effects acting through leukocytes if studied in the context of a full cellular immune response.

Transforming Growth Factor- β Has Cancer-Promoting and -Inhibiting Effects

TGF- β is one of the key players involved in the communications between CAFs and carcinoma cells, but again is expressed by multiple cell types, including the stromal fibroblasts, the inflammatory cells, and carcinoma cells (55). Whereas FSP1 and CXCL12 clearly are promoters of carcinogenesis, TGF- β is a factor with much more complicated effects on tumorigenesis. TGF- β is immunosuppressive

when acting on inflammatory cells, thereby promoting carcinogenesis through inhibition of the immune response against the neoplasm. However, when acting on the epithelium, TGF- β is growth inhibiting and thus brakes carcinogenesis until the carcinoma cells overcome its growth suppressive effects (Figure 17-5). At that point TGF- β becomes a stimulator of metastasis (reviewed in [55]). TGF- β 1 can induce differentiation of resting fibroblasts into myofibroblasts in culture (reviewed in [56]) and increases FSP1 expression. Increased secretion of TGF- β in irradiated mammary stroma may be part of the mechanism by which irradiated stroma stimulate tumorigenesis [15]. Finally, overexpression of TGF- β by fibroblasts stimulates neoplastic growth of human breast epithelium *in vivo* (11). Thus, TGF- β is a key player in the generation of a reactive stroma and its action.

The actions of TGF- β are clearly cell type-specific: When the TGF- β receptor II is genetically removed from fibroblasts in mice, rendering these fibroblasts unresponsive to TGF- β signaling, the mice develop neoplasias and carcinomas without any further genetic manipulations of the epithelium (8). However, ablation of the TGF- β receptor II in epithelial cells inhibits tumor progression (57). These results suggest that TGF- β acting on the fibroblasts normally protects the epithelium from developing into carcinomas, whereas TGF- β secreted by CAFs and acting on the epithelium promotes carcinogenesis.

Type I Collagen and Cancer Progression

In carcinoma progression, the CAFs are largely responsible for the desmoplastic response, which is a strong stromal response characterized by pronounced changes in the ECM, including increased amounts of collagens, fibronectins, proteoglycans, and glycosaminoglycans (56). Many carcinomas, including human breast cancer, show a remarkable upregulation of fibrillar collagen and collagen-associated proteins. In fact, some of changes in the composition of the ECM may occur before the carcinoma evolves: High mammographic breast density is a strong predisposing factor for the development of sporadic breast cancer and confers a risk of about 4 relative to women with fatty breasts (58). Mammographic density is reflective of a changed stromal microenvironment, including increased amounts of collagen (59). Many stromal effects on normal development and tumor development of the mammary gland are shared (60). Type I collagen is a classic substrate of the matrix metalloproteinases (MMPs), a family of proteolytic enzymes identified as modifiers of mammary carcinogenesis (reviewed in [25]). Cleavage of collagen by MMPs is an important step in tumor invasion through basement membranes and type I collagen (61). It is noteworthy that the presence of collagen-dense fibrotic foci within mammary carcinomas correlates with an adverse prognosis (62) and that increased expression of collagen type I is associated with increased risk of metastasis and decreased survival in many human cancers, including breast, lung, and prostate cancers (63).

How collagen contributes to the development and progression of cancer is not known. However, it is known that cancer cells are influenced by the ECM. For example, the sensitivity of cancer cells to apoptotic stimuli are regulated by interactions between integrin receptors on the cancer cells and proteins in the ECM (64). Furthermore, malignant transformation of the breast is associated

with dramatic changes in gland tension that include increased ECM stiffness, elevated compression forces, and high tensional resistance stresses. These changes perturb tissue morphogenesis and facilitate tumor invasion (65,66). Interestingly, overexpression of lysyl oxidase-related protein-1 (LOR-1), a protein that is involved in the cross-linking and thereby stabilization of the collagen fibers, results in the formation of very dense collagen fibers surrounding the tumors (67). However, rather than preventing invasion through the encapsulation of the carcinoma, the LOR-1-overexpressing cells become highly invasive (67). Similarly, lysyl oxidase contributes to hypoxia-induced metastasis of tumors (68).

In addition to any direct effects on the cancer cells, collagen may play a role in the regulation of leukocyte behavior within tumors. Indeed, there may be cross-talk between the collagen-rich stroma and the infiltrating leukocytes in tumors: Macrophages and dendritic cells become activated and secrete chemokines in response to binding to type I collagen (69). Vice versa, leukocytes produce the ECM protein SPARC, which determines stromal collagen deposition in carcinomas. In the absence of leukocyte-produced SPARC, tumors have reduced growth, and large areas of necrosis and impaired vascularization are observed (70).

Remodeling of the Tumor Microenvironment by Matrix Metalloproteinases

The ECM surrounding the cancer cells is influenced by proteases that are active there. One example would be the MMPs, which represent a family of ECM and membrane-bound proteases that cleave many substrates, including ECM components and chemokines. They are important effectors of the altered tumor microenvironment and promote cancer progression by stimulating tumor growth and proliferation, regulating apoptosis, inflammation, angiogenesis, invasion, and metastasis. They are overexpressed in most types of cancer and correlate with advanced tumor pathology. In fact, an increase in their expression and activity often correlates with tumor angiogenesis, metastasis, and poor prognosis (reviewed in [25,71]). However, most MMPs are not expressed by the cancer cells themselves and instead are expressed and activated in the stroma (25,71).

The remodeling of the stromal microenvironment, for example, the cleavage of type I collagen, is mediated in part by secreted proteases, including MMPs (25). Both MMPs and collagen type I are present in tumors, and together collagen turnover may mobilize growth factors bound in the matrix or release collagen fragments that promote cancer cell survival. The collagenolytic MMPs include MMP-1, MMP-8, MMP-13, MT1-MMP, and MT2-MMP.

MMP-13 is expressed by CAF-like cells in human breast cancer (72), and *in vitro*, breast cancer cells can stimulate fibroblasts to secrete MMP-13 (73). However, MMP-13 may also be expressed by lymphocytes and macrophages (74,75). Substrates for MMP-13 *in vitro*, including TGF- β , CXCL12, and type I collagen (25). Latent TGF- β is cleaved and activated by MMP-13 (76). CXCL12 is cleaved and inactivated by MMP-13 (77), and type I collagen is cleaved into specific fragments by MMP-13. Thus, MMP-13 is a factor secreted by CAFs that may regulate the activity of other factors secreted by or

acting on the CAFs, complicating the interpretation of the role of the individual factors in carcinogenesis. In addition to the examples with MMP-13 and its CAF-secreted substrates, TGF- β 1 can up-regulate the CXCL12 receptor CXCR4 (78); TGF- β 1 and type I collagen can stimulate FSP-1 protein expression (79), and FSP1 can stimulate endothelial cells to up-regulate the expression of MMP-13 (51). Thus, stromal factors and stromal cells are coconspirators and may act additively, synergistically, or repress each other's functions (Figure 17-4).

Matrix Metalloproteinases Promote Carcinogenesis

Epithelial-to-mesenchymal transition is characterized by loss of adhesion between cells, decreased expression of epithelial markers (e.g.,

E-cadherin), increased expression of mesenchymal markers (e.g., vimentin), and increased motility. Although EMT is a normal developmental process, it may also promote cancer progression. MMPs can promote carcinogenesis through triggering EMT in the cancer cells and by generating intracellular genomic instability (reviewed in [80]).

An example of an MMP that promotes carcinogenesis is MMP-3/stromelysin-1. MMP-3 itself is not expressed in epithelial cells, but instead is expressed by the surrounding stromal cells (81). MMP-3 promotes mammary hyperplasias and cancer in mice (7,82), and it is up-regulated in human breast cancer (25). In the mammary gland, MMP-3 promotes proliferation and branching in ductal epithelial cells, but it induces apoptosis in alveolar epithelial cells (83–86). Consistent with its role in tumor initiation and progression, overexpression of MMP-3 alters epithelial cell adhesion by

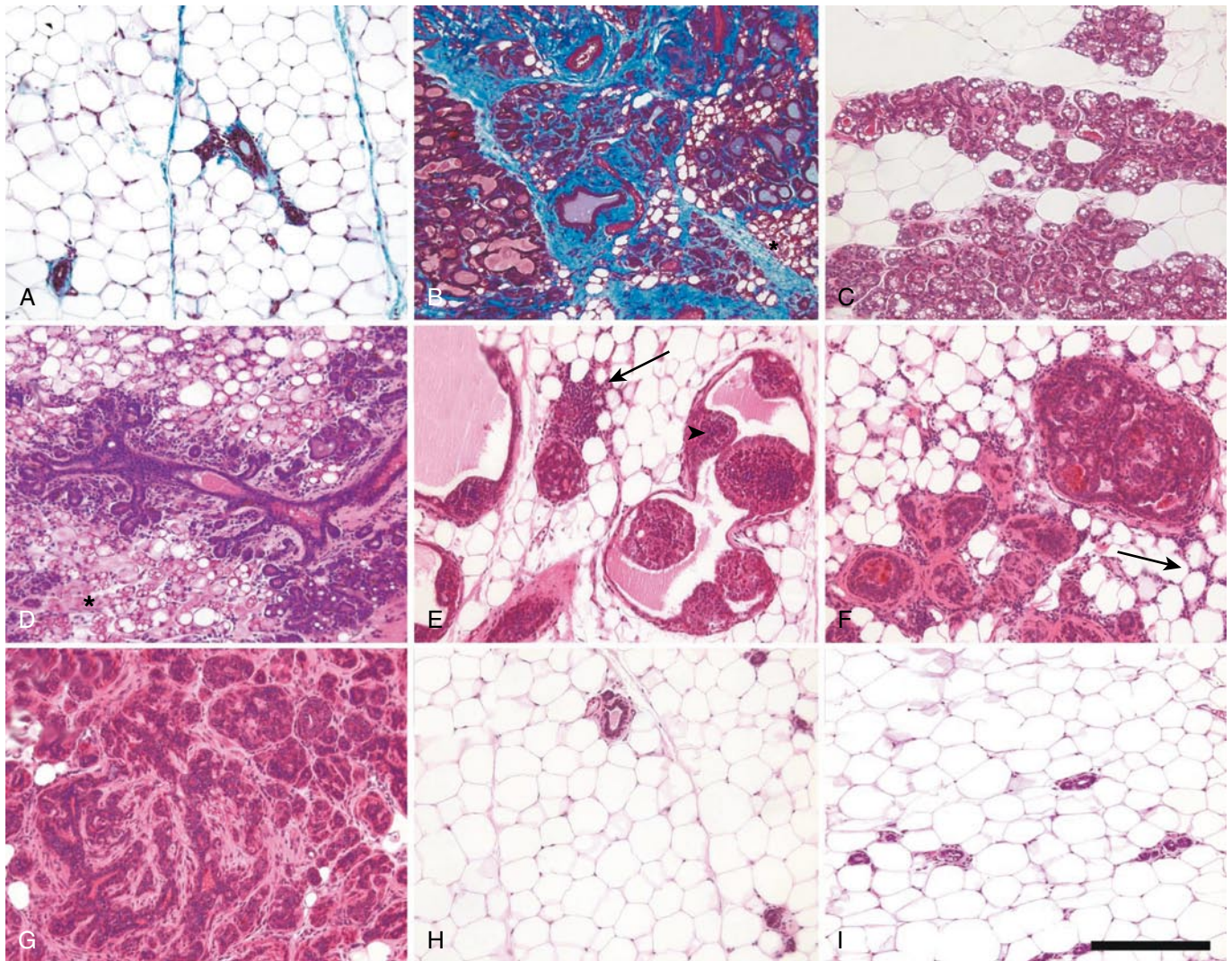


FIGURE 17-6 MATRIX METALLOPROTEINASE-3 (MMP3) INDUCES TUMORS IN THE MAMMARY GLAND. Histologic sections are from (A) nontransgenic, (B–G) *WAP-MMP-3* transgene-expressing, (H) *WAP-MMP-3* transgene-nonexpressing, and (I) *MMP-3;TIMP1* double transgene-positive female mice.

A: Normal mammary gland with resting ducts (Du), abundant adipose tissue (asterisk), and minimal periductal (Pd) and septal (S) collagen (blue). **B:** Severe hyperplasia (Hp) with considerable intervening fibrosis (Fb; blue) and multilocular adipocytes (asterisk). **C:** Hyperplastic alveolar nodule (HAN) with lipid droplets characteristic of secretory activity even though this gland comes from a nulliparous mouse. **D:** Multifocal alveolar Hp with eosinophilic (pink) fibrotic areas and multilocular adipocytes (asterisk). **E:** Intraductal papillary Hp with lymphocytic infiltrates (Ly). The small hyperchromatic cells (Me) were cytokeratin-8 negative and smooth muscle actin positive, indicating the abnormal presence of myoepithelial cells within the severely distended ducts. **F:** Atypical hyperplasia (AH) with lymphocytic infiltrates (Ly) and mild fibrosis (Fb). **G:** Atypical hyperplasia with considerable fibrosis. **H,I:** Normal mammary histology seen with the loss of *Str1* transgene expression or its inhibition by *TIMP1*, respectively. **A,B:** Masson trichrome stain; (**C–I**) hematoxylin and eosin stain; scale, 200 μ m. (From Sternlicht MD, et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 1999;98:137–146, with permission.)

cleaving E-cadherin, inducing EMT, and promoting premalignant and malignant lesions as well as genomic instability in the mammary gland (Figure 17-6; 82,87). Altering adhesion of epithelial cells leads to reduced levels of p53 and increases DNA damage (88,89).

A clue to the mechanism by which MMP-3 and altered adhesion triggers these events comes from the observation that MMP-3 induces Rac1b, an alternatively spliced variant of Rac1, which then stimulates increased levels of mitochondrial reactive oxygen species (ROS) and thereby DNA oxidative damage (87). These ROS are required to trigger the EMT and DNA damage (87).

MMP-3 is not unique in inducing tumors. Overexpression of MMP-7 (90) and MT1-MMP (MMP14; 91) also leads to mammary tumors in mice. MT1-MMP has been shown to play a role in genomic instability. Its overexpression leads to cleavage of pericentrin, a centrosomal protein, and the cells are polyploidy with abnormal spindles (92). Interestingly, cytokinesis defects in p53-negative primary mouse mammary epithelial cells have non-random genomic amplifications, including the region containing MMP genes (93).

Prospects for Microenvironment Cells as Prognostic Indicators of Cancer and Potential Drug Targets

The complex signaling networks within the cancer cells have long been studied and targeted for drug development. As research

efforts expand to study the microenvironmental cues and effects, the complex signaling *between* the cells in the tumor tissue is a central focus of cancer research. The ability of the stromal cells to regulate epithelial carcinogenesis makes them potential drug targets. However, we are still far from developing strategies to restore aberrant signaling between the stroma and the epithelium in carcinomas (6). Nevertheless, a few drugs that target the stromal influence on carcinogenesis, for example angiogenesis inhibitors, are showing significant effects on cancer patient survival proving that targeting the stroma is a feasible direction for cancer treatment (94). Genes expressed by the stromal cells in the tumors are promising prognostic predictors in human breast cancer (95). As the molecular basis for the influence of fibroblast-like cells, CAFs, TAMs, and other innate immune cells on epithelial cancers emerge, they may point to new targets for therapy.

Acknowledgments

Supported by grants from the National Institutes of Health (ES012801, CA072006, CA057621, and CA105379; Z.W.) and fellowships from the American Cancer Society (L.E.L.), the Ruth Kirschstein National Research Service Award (CA103534; L.E.L.), the California Breast Cancer Research Program (M.E.) and the Danish Medical Research Council (M.E.).

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Tumor Angiogenesis

Solid tumors require a vascular system to grow beyond ≈ 2 mm in diameter, a size at which diffusion of oxygen and nutrients is limiting. The establishment of a tumor vasculature through the process of angiogenesis overcomes these limitations, while also providing a conduit through which cancer cells can metastasize. Judah Folkman's proposal in 1971—that cancer progression might be inhibited, or even reversed, by blocking tumor angiogenesis (1)—sparked a remarkable flurry of activity in basic and clinical research. A milestone in angiogenic cancer therapy was passed in 2004, when the U.S. Food and Drug Administration (FDA) approved the anti-angiogenic monoclonal antibody Avastin (bevacizumab) as a first-line treatment for metastatic colorectal cancer. A large number of other anti-angiogenic drugs, as well as modified chemotherapeutic regimens that may block tumor angiogenesis, are in phase 2 and phase 3 clinical trials (<http://www.cancer.gov/clinicaltrials/developments/anti-angio-table>). This chapter describes our understanding of tumor angiogenesis, the specific molecular pathways that regulate this process and the different therapeutic approaches currently under development and testing.

The close association between tumor growth and increased vascularity was described in the nineteenth century by several researchers (2), including the pathologist Rudolf Virchow, who also first proposed a link between chronic inflammation and cancer (3). An important experimental advance in angiogenic research came in the 1920s, when transparent chambers were first used to observe the growth of vessels into tumors in animals in real time. This intravital technique is still widely used today with modern imaging methods and overcomes some of the limitations inherent to studying vascular beds in tumor samples isolated and fixed postmortem (2). In a seminal study published in 1945, Algire and colleagues (4) used transparent chambers to follow vessel recruitment to a variety of normal and malignant tissues transplanted into mice. Their studies provided some of the first observations that transplanted tumor tissue, in contrast with normal tissue, induced the development of an extensive vascular bed; moreover, this angiogenic response preceded rapid tumor growth. The authors succinctly stated the now-axiomatic idea that “the rapid growth of tumor explants is dependent on the development of a rich vascular supply” (4).

The correlation between tumor angiogenesis and growth led to an extensive search for specific angiogenic molecules produced by tumors. A large number of studies in different species over the last 20 years have identified a rapidly growing list of molecular signaling pathways that promote and regulate vessel formation both in embryogenesis and in pathophysiological settings. These pathways provide potential molecular targets for anti-angiogenic therapies to treat cancer and other vascular diseases, including macular degeneration, diabetic retinopathy, and arthritis (5,6). The critical cellular targets of these therapies are **vascular endothelial cells (ECs)** and supporting mural cells or **pericytes (PCs)** that are recruited from surrounding healthy tissue, or derived from the bone marrow, to form new vessels in the growing tumor. It has been suggested that targeting these “normal” cells obviates the problem of acquired drug resistance common to genetically unstable cancer cells. One of the key ideas behind anti-angiogenic strategies is that of the **angiogenic switch**, which is generally defined as the point at which the balance between naturally occurring pro- and anti-angiogenic factors is tipped to favor vascular development (7). Extensive studies in different experimental models, as well as correlative clinical data, strongly support this concept. Interestingly, the stage of tumor progression at which an angiogenic switch occurs can vary. Many tumors induced experimentally in mice fail to grow beyond a few millimeters in diameter until they recruit new blood vessels (7). In contrast, human astrocytomas initially grow as a cuff of living cells surrounding existing blood vessels and trigger angiogenic responses only late in their progression to glioblastomas (8). The experimental and clinical support for the angiogenic switch model has prompted many researchers to search for natural angiogenesis inhibitors, with the hope that increasing their expression or function will result in tumor arrest or regression (9). We will return to this topic in subsequent sections of this chapter.

The following three sections will describe the development of vascular structures in normal and malignant tissues, the signaling pathways that regulate their formation, and the therapeutic strategies that underlie ongoing drug development and clinical testing. Finally, we will discuss some of the remaining questions and challenges that are likely to drive angiogenic research in the coming years.

Vascular Development

Development of the vascular system is one of the first events in embryonic organogenesis. Initially, vascular networks form independently in the yolk sac and the embryo and then connect to generate a closed circulatory system. In a process known as **vasculogenesis**, endothelial cell progenitors (**angioblasts**) and their derivative ECs aggregate in the yolk sac to form a primitive vascular network or plexus, composed of an interconnected series of ECs organized into tubular structures of approximately uniform dimensions (Figure 18-1). Subsequent **angiogenesis** occurs through vessel **sprouting**, in which ECs from existing vessels respond to angiogenic signals by degrading their basement membrane, loosening their association with support cells, altering their morphology, and proliferating. These ECs migrate in response to chemotactic signals and coalesce to form new vessels that connect to the existing vasculature. The coordinated recruitment of pericytes and smooth muscle cells results in vessel maturation. In a parallel mechanism termed “intussusception,” columns of endothelial

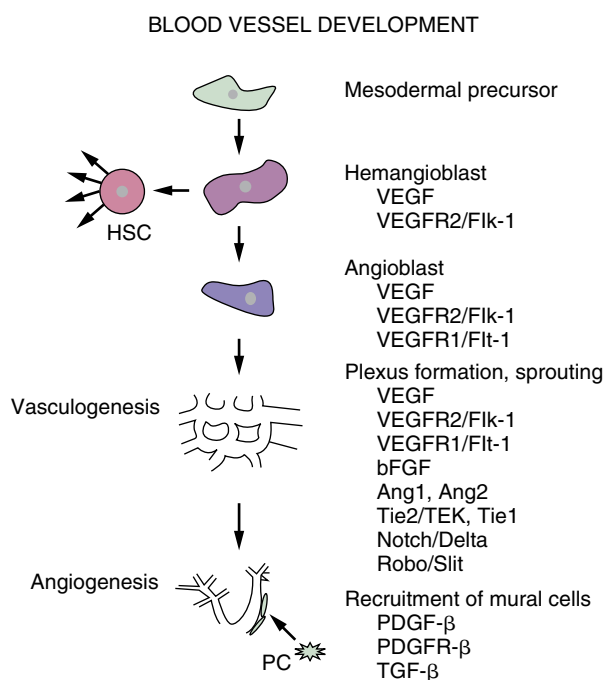


FIGURE 18-1 MAJOR EVENTS IN VASCULAR DEVELOPMENT. Some of the critical signaling molecules and receptors are shown in red corresponding to the cells or processes in which they are known to play a role. Vascular progenitors are derived from vascular endothelial growth receptor-2 (VEGFR2/Fik-1)-positive cells in the lateral plate mesoderm. These bipotential hemangioblasts give rise to haematopoietic stem cells (HSCs) and vascular endothelial precursors (angioblasts). In the yolk sac, angioblasts align to generate a primary capillary plexus (vasculogenesis). Vessels in this plexus grow primarily by sprouting, which involves endothelial cell proliferation and migration (angiogenesis), and eventually connect to vessels in the embryo to form a closed vascular system. Vasculogenesis and angiogenesis are both highly dependent on VEGF, angiopoietins, and their receptors, along with many other signaling molecules (see figure and text). Maturation of the vascular system requires remodeling of the vascular network into large and small vessels, along with the recruitment of supporting mural cells (pericytes and smooth-muscle cells). PC, pericyte; Ang, angiopoietin; Tie2/Tek and Tie1, Tie family of endothelial receptor tyrosine kinases; Notch/Delta, Notch receptor/Delta, ligand; Robo/Slit, roundabout receptor/slit ligand; PDGF-β, platelet-derived growth factor B; PDGFR-β, PDGF receptor B; TGF-β, transforming growth factor-β. (From Risau W. *Mechanisms of angiogenesis*. *Nature* 1997;386:671–674, with permission.)

cells create a barrier in the lumen of a preexisting vessel, thus partitioning it into multiple independent vessels (Figure 18-2; 5,10). This complex series of events produces a closed, highly arborized system of larger and smaller vessels including arteries, veins, and capillaries. In contrast to the yolk sac, angioblasts in the embryo migrate along specific pathways and aggregate directly to form the dorsal aorta and posterior cardinal vein, without passing through an intermediate plexus phase (11). These vessels undergo subsequent remodeling and ultimately connect to the extra-embryonic yolk sac vessels to form a mature vascular system. Interestingly, vascular development is also intimately connected to development of the hematopoietic lineages, as angioblasts and hematopoietic stem cells are thought to arise from a common **hemangioblast** precursor (Figure 18-1; 11,12).

The vessels of the **lymphatic** system collect and return interstitial fluids, particulates, and extravasated cells to the venous circulation. Lymphatic vessels differ from blood vessels in that lymphatic capillaries have internal membranous valves that prevent fluid backflow and are not surrounded by support cells (13). Lymphatic ECs are derived from primitive veins, and express and respond to a different spectrum of receptors and signaling molecules than ECs in blood vessels (13; see following section). The ability of cancer cells to invade lymphatics and collect in lymph nodes, complex organs involved in local immune surveillance, is a classic measure of tumor metastasis. It is likely that the lymphatic vessels at the periphery of a solid tumor are most directly involved in metastasis, as interstitial pressure within the tumor often leads to vessel collapse (5,13). Research evidence supports the idea that lymphatic ECs may secrete chemokines that attract tumor cells and may therefore participate more actively in metastasis than was previously recognized (14).

Over the past 15 years, work from many laboratories has demonstrated that vascular development in normal tissues is under elaborate genetic control. Intriguingly, many of the molecules that regulate developmental angiogenesis have also been shown to drive angiogenesis in cancer and other pathophysiologic conditions, although their expression and function in tumors are often highly uncoordinated. These factors and their activity in tumor angiogenesis are discussed in detail later. It is also important, however, to appreciate that angiogenesis is not regulated solely by hard-wired genetic programs. Local physiologic conditions, particularly oxygen deprivation (**hypoxia**), have profound effects on the expression of angiogenic molecules. Hypoxia represents an important angiogenic signal in rapidly growing embryonic tissues and in tumors (15). In addition, severely hypoxic conditions are thought to protect tumor cells from radiation therapy, which depends on the generation of reactive oxygen intermediates to kill tumor cells. Finally, hypoxic regions in tumors appear to select for highly malignant cancer cells (16). The stabilization of **hypoxia-inducible transcription factors (HIFs)** in normal tissues and in tumors stimulates the expression of **vascular endothelial growth factor (VEGF)** and other angiogenic molecules, and several treatment strategies based on interfering with HIF activity are being developed (Figure 18-3; 18,19).

Localized angiogenesis is also an important aspect of normal wound healing. Critical angiogenic signals are provided by **inflammatory cells** including macrophages, neutrophils, and

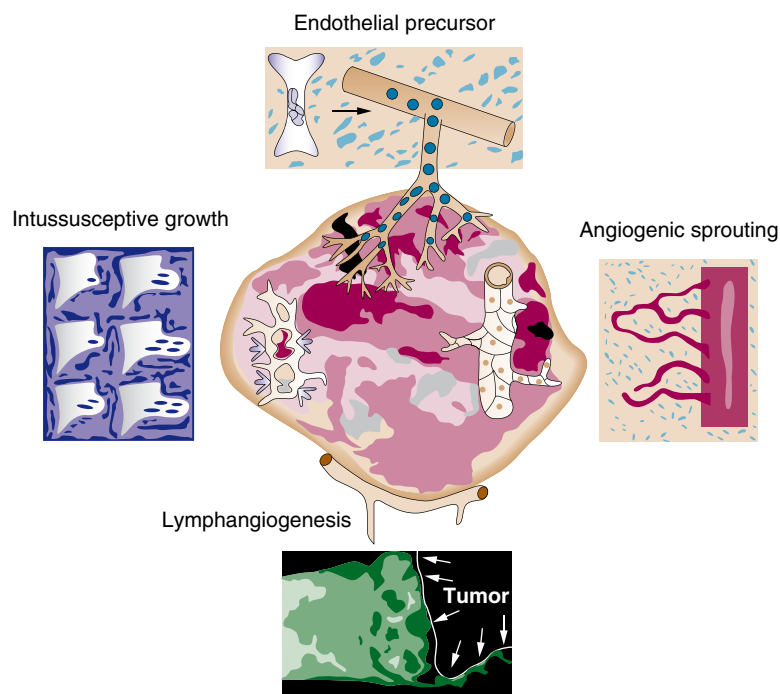


FIGURE 18-2 CELLULAR MECHANISMS OF TUMOR ANGIOGENESIS. Tumor vessels grow by multiple mechanisms, some of which are formally similar to those observed in normal vascular development: (1) budding of endothelial sprouts and formation of bridges (angiogenesis) and (2) insertion of interstitial tissue columns into the lumen of pre-existing vessels (intussusception). In contrast with normal vascular development, the signaling events controlling these events are often highly disordered, resulting in chaotic vascular organization, uneven blood flow, and localized hypoxia. In addition, endothelial cell precursors home to tumors from the bone marrow or peripheral blood (3) where they can contribute, directly or indirectly, to the endothelial lining of tumor vessels. Lymphatic vessels (4) around tumors drain interstitial fluid and also provide a gateway for metastasizing tumor cells. (From Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249–257, with permission.)

mast cells recruited to wounds and activated resident fibroblasts. Upon remodeling and fusion with the surrounding vasculature, these new vessels restore normal blood supply to the affected area. Infiltrating inflammatory cells and fibroblasts often make up a large bulk of solid tumors and also produce or release angiogenic

factors, but do so in a highly disordered and unregulated manner, thereby contributing to persistent and disorganized tumor angiogenesis (3). Research studies have demonstrated that genetic inactivation of tumor-associated macrophages greatly reduces tumor angiogenesis and metastasis in mouse cancer models (20).

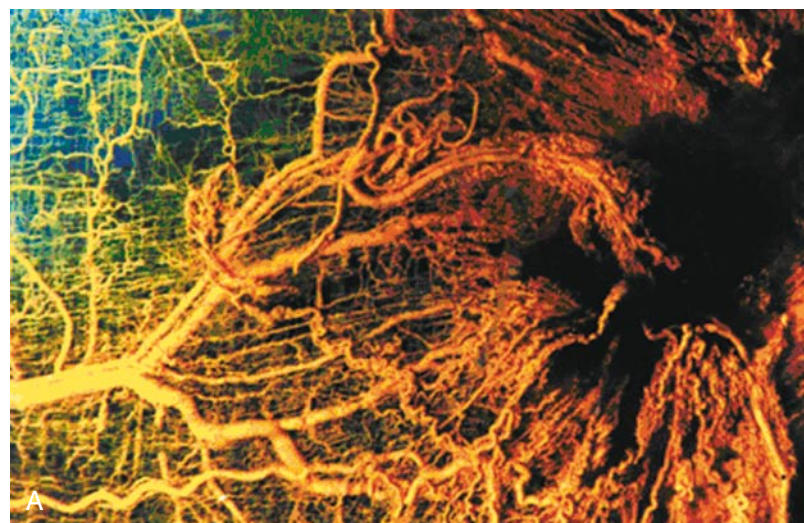
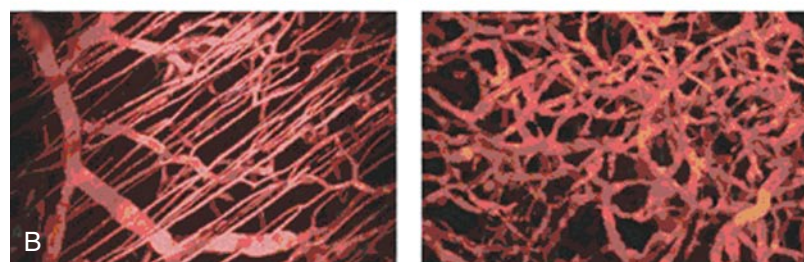


FIGURE 18-3 The highly disorganized nature of tumor vasculature can be visualized by generating a polymer cast before fixation (A), or using intravital imaging techniques that reveal functional vessels in live tissues (B). As opposed to the clearly ordered arrangement of vessels in normal tissue, the chaotic nature of tumor vessels reflects the disrupted balance of pro- and anti-angiogenic factors generated by tumor and stromal cells. (From Ref. 17, with permission.)



Normal tissue

Tumor

In adult humans and mice, there is little active angiogenesis, with the notable exceptions of the female reproductive system and general wound healing. Nevertheless, rapid growth of any tissue (neoplasias, adipose tissue, regenerating liver, etc.) invariably requires a supply of oxygen, nutrients, and hormones and is typically accompanied by angiogenesis. Consequently, angiogenesis can be seen as a genetically programmed, dynamic process that can be activated in nearly any tissue in response to local stimulatory signals. The fact that most blood vessels in the adult body are quiescent has been proposed as an advantage for anti-angiogenic strategies, as it would predict that such drugs would be less generally toxic than other cytotoxic chemotherapies or radiation therapy. In a complementary fashion, some authors have proposed using pro-angiogenic molecules to stimulate localized angiogenesis to repair ischemic tissue damage or to normalize blood flow in neoplasias and thereby sensitize them to radiation therapy (21,22).

Tumor Vasculature

The blood vessels found in solid tumors tend to be highly disorganized compared with those of normal organs and are characterized by tortuous and misshapen vessels that sometimes terminate in open-ended blood lakes (Figure 18-3)(5,23). Microscopic analysis of tumor vessels reveals disrupted junctions between tumor ECs and reduced or inconsistent coverage by pericytes, which helps explain the increased permeability characteristic of tumor vessels (24). The origin of some tumor ECs is also controversial: in addition to ECs recruited through sprouting of preexisting vessels, growing evidence supports a role of circulating endothelial progenitor cells (CEPs) that differentiate into endothelial-like cells or promote expansion of bona fide ECs (Figure 18-2). In mice, the degree to which CEPs contribute directly to the lining of new tumor vessels varies considerably, depending on the model used, genetic background, and other factors (25). In addition, bone marrow-derived myeloid cells contribute to tumor angiogenesis; these cells have been reported to express a variety of cell surface markers, including those common to endothelial cells (Tie-2) and myeloid cells (CD11b), and may function by providing paracrine angiogenic signals (26,27). It is interesting to note that genetic ablation of bone marrow-derived, Tie-2-expressing monocytes (TEMs) has profound effects on tumor angiogenesis in mice (28).

As a consequence of altered tumor architecture, tumors often have sluggish, uneven, and highly variable patterns of blood flow (23) and direct arteriole-venule shunts (29). Tumor vessels also differ from normal vasculature in being exposed to an acidic microenvironment characterized by oxygen and nutrient deprivation. In rapidly growing tumors, or those with leaky vessels, high interstitial pressure can lead to vessel collapse, resulting in localized anoxic and/or ischemic regions. This typically results in pockets of necrotic cell death surrounded by a penumbra of hypoxic, but living cells. This hypoxic environment can induce the expression of VEGF and other angiogenic molecules in tumor cells and ECs, thereby stimulating angiogenic growth and remodeling (5). In addition, tumor ECs often alter the spectrum of **integrin** proteins expressed on their surface, inducing $\alpha_v\beta_3$ and $\alpha_v\beta_1$ integrin expression in particular. When bound to insoluble extracellular

substrates such as fibronectin or vitronectin, $\alpha_v\beta_3$ stimulates VEGF-dependent tumor angiogenesis: in contrast, unligated $\alpha_v\beta_3$ can promote apoptosis in tumor ECs²⁹.

The extent to which tumors generate vascular beds is often expressed as **microvessel density (MVD)**, which can vary widely within a given tumor and between tumors of similar or different tissues. MVD is determined empirically by staining tumor sections with antibodies raised against proteins expressed on ECs, including CD31 (PECAM), CD34, and von Willebrand factor. Many clinical studies have demonstrated that MVD is a highly useful prognostic indicator for a wide array of cancers, including breast, prostate, non-small cell lung, gastrointestinal, and even hematologic tumors (31). It may seem surprising, therefore, that MVD is not necessarily an accurate measure of angiogenesis-dependent tumor growth or a reliable indicator of anti-angiogenic therapeutic efficacy. Because tumor cells and/or associated stroma may overexpress VEGF or other pro-angiogenic molecules, MVD may greatly exceed the basic metabolic requirements of a growing tumor. In some cases, however, MVD may actually be lower in rapidly growing tumors than in the corresponding normal tissue. The striking heterogeneity of functional vessels within a tumor, and the ability of many cancer cells to withstand severe hypoxia, glucose deprivation, and tissue acidity, makes it difficult to assess the effects of anti-angiogenic therapies based solely on MVD (31).

Critical Signaling Factors: Targets for Therapy

A growing list of signaling molecules has been shown to regulate different aspects of developmental and pathologic angiogenesis. Primary among these is the family of VEGFs, which, along with their receptors, regulate endothelial cell proliferation, survival, and function. The vascular-specific **angiopoietins** and their receptor tyrosine kinases also play important roles in angiogenic remodeling. In addition, vascular development is regulated by signaling pathways familiar from other developmental processes, including fibroblast growth factors (FGFs; in particular, basic or **bFGF**), transforming growth factor beta (**TGF- β**), **Notch** and its ligand **Delta4**, and platelet-derived growth factor (**PDGF**). A number of molecules originally implicated in controlling axon guidance, including the **semaphorins**, **netrins**, and **Robo/slit**, have been shown to contribute to vascular development (11,32). Finally, the Notch pathway, along with the **EphB4/ephrinB2** signaling system, has been shown to control specification of arteries and veins (11,32). Our understanding of the mechanisms by which these genes and pathways regulate angiogenesis is based largely on genetic “knock-out” experiments in mice, often confirmed by in vitro cell-based assays or in experimental tumors. How this complex array of signaling pathways is coordinated to regulate angiogenic events in normal organogenesis and disease is a focus of intensive research. The discovery of endogenous angiogenic inhibitors, including **thrombospondin-1**, **endostatin**, **tumstatin**, and others, provided strong evidence to support the idea that angiogenesis is a function of the balance between pro- and anti-angiogenic factors (9). In this section, we will discuss the molecular biology and function

of a small subset of pro-angiogenic and anti-angiogenic factors that show particular promise as targets for cancer therapies.

Pro-Angiogenic Factors

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (also known as VEGF-A) is among the most potent angiogenic factors described and stimulates EC proliferation, survival, chemotaxis, and vessel permeability. VEGF belongs to a family of structurally related growth factors that includes placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D. VEGF is a homodimeric glycoprotein of 45 kD and is expressed in four different molecular weight forms, VEGF-121, VEGF-165, VEGF-189, and VEGF-206, produced by differential mRNA splicing. VEGF-121 is diffusible, whereas the other forms bind to heparin and heparin proteoglycans in the extracellular matrix (ECM) and on cell surfaces. These bound forms are released through the action of proteases, including plasmin and matrix metalloproteases (MMPs), which are produced by tumor cells and/or by activated stromal cells. Interestingly, VEGF was first identified as VPF (vascular permeability factor) on the basis of its ability to increase the leakage of fluid and plasma proteins from blood vessels (2,5,8,21,24). These leaked proteins provide an ECM for migrating ECs, and their release into interstitial spaces represents an early step in angiogenesis. The central importance of VEGF in regulating angiogenesis became clear through genetic targeting experiments in mice. Loss of only one allele of VEGF resulted in lethality at embryonic day 9.5 (E9.5), characterized by a reduction in ECs and abnormal vessel morphology (33,34). Embryos lacking both VEGF alleles died even earlier (E8.5) and displayed a complete absence of the dorsal aorta and other vascular structures.

VEGF mediates its effects by binding its cognate receptor tyrosine kinases, VEGFR-2 (also called Flk-1 or KDR) and VEGFR1 (also called Flt-1). Binding of VEGF to VEGFR-2/Flk-1 triggers receptor autophosphorylation and robustly activates several downstream signaling pathways (including phosphoinositide-3-kinase (PI3K), Src, and protein kinase C [PKC]) leading to rapid and profound effects on EC proliferation, survival, migration, and gene expression (11,35). Genetic ablation of Flk-1 in mice caused embryonic lethality at E8.5 that correlated with a loss of normal vascular structures and hematopoietic cells, providing indirect support for the existence of a bipotential **hemangioblast** precursor cell (36,37). Subsequent studies have confirmed the importance of VEGF and VEGFR-2/Flk-1 in hematopoietic development (11). Although VEGFR-1/Flt-1 also binds VEGF, its major angiogenic function may be to modulate the amount of VEGF available to bind to VEGFR-2/Flk-1 (24,38). Deletion of the gene encoding murine VEGFR-1/Flt-1 resulted in embryonic lethality; however, this lethality was rescued by transgenic expression of the extracellular domain of VEGFR-1/Flt-1 alone, with no cytoplasmic signaling domain. Although these results argue strongly that VEGFR-1/Flt-1 acts as a nonsignaling sink for free VEGF, more recent studies indicate that it can, in fact, modulate pathophysiologic angiogenesis, possibly by intermolecular phosphorylation of VEGFR-2/Flk-1 (38). Neuropilins 1 and 2 can

also act as a sink for VEGF, and appear to function, at least in part, by presenting VEGF to VEGFR-2/Flk-1 or by modulating its effective free concentration (39).

The central role of VEGF signaling in tumor angiogenesis has been clearly demonstrated in a wide variety of experimental models, including VEGF overexpression in tumor or host cells, treatment with recombinant VEGF, increased VEGF expression in response to oncogene activation, or inhibition by antisense VEGF oligonucleotides or anti-VEGF antibodies (2,32). In an elegant genetic experiment, Johnson and colleagues inactivated the VEGF gene in transformed mouse embryonic fibroblasts (MEFs), which were then used to generate subcutaneous fibrosarcomas in immunocompromised mice. Compared with wild-type controls, fibrosarcomas lacking VEGF expression were greatly reduced in size, displayed dramatically lower vascular density and permeability, and had higher levels of tumor cell apoptosis (40).

Many oncogenes (including Kras, Her2, FOS, and trkB), tumor suppressors (including pVHL and p53), and growth factors (including PDGF, bFGF, and TGF- β) promote angiogenesis, in part by inducing the expression of VEGF, directly or indirectly (32). The von Hippel Lindau (VHL) tumor suppressor is a particularly interesting case in point. Patients with VHL disease, a hereditary cancer syndrome, develop a variety of tumor types including highly vascularized renal clear cell carcinomas, cerebral hemangioblastomas, and retinal hemangiomas. The pVHL protein functions as an E3 ubiquitin ligase, which targets the hypoxia-inducible factors (HIFs) HIF-1 α and HIF-2 α for degradation via the 26S proteasome (41). HIF-1 α and HIF-2 α are known to directly bind to the *Vegf* gene promoter and activate its transcription in hypoxic cells (Figure 18-4). When pVHL expression or function is lost, cells can no longer degrade the HIF- α subunits under conditions of abundant oxygen, leading to constitutive expression of VEGF and other target genes, thereby promoting tumor angiogenesis in some cases (Figure 18-5). The close spatial overlap between HIF- α protein accumulation and VEGF expression in hypoxic tumor cells is a further indication that HIF-dependent VEGF expression is an important aspect of tumor angiogenesis (Figure 18-3). Both HIF-1 α and HIF-2 α can activate VEGF expression independently, hence deletion of either subunit has relatively subtle effects on embryonic VEGF expression, despite the fact that both mutations are embryonically lethal (15,42–44). Targeted deletion of the common binding partner (HIF-1 β or ARNT), however, resulted in early embryonic lethality with substantial loss of VEGF expression (45) associated with fundamental defects in angiogenesis (46). The close link between the HIFs and VEGF expression in tumors has prompted the design of specific HIF inhibitors, with a view toward limiting expression of VEGF and other hypoxically induced angiogenic factors in cancer and other diseases (18,19).

There are several VEGF homologues in mammals, including VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). Each of these has different influences on angiogenesis and binds to one or more of the family of VEGF receptors. VEGF-C and VEGF-D regulate lymphangiogenesis through their effects on VEGFR-3/Flt-3, which is expressed on lymphatic ECs (13). PlGF binds to both VEGFR-1/Flt-1 and the neuropilins, displacing it and thereby making it available for binding to VEGFR-2 (39).

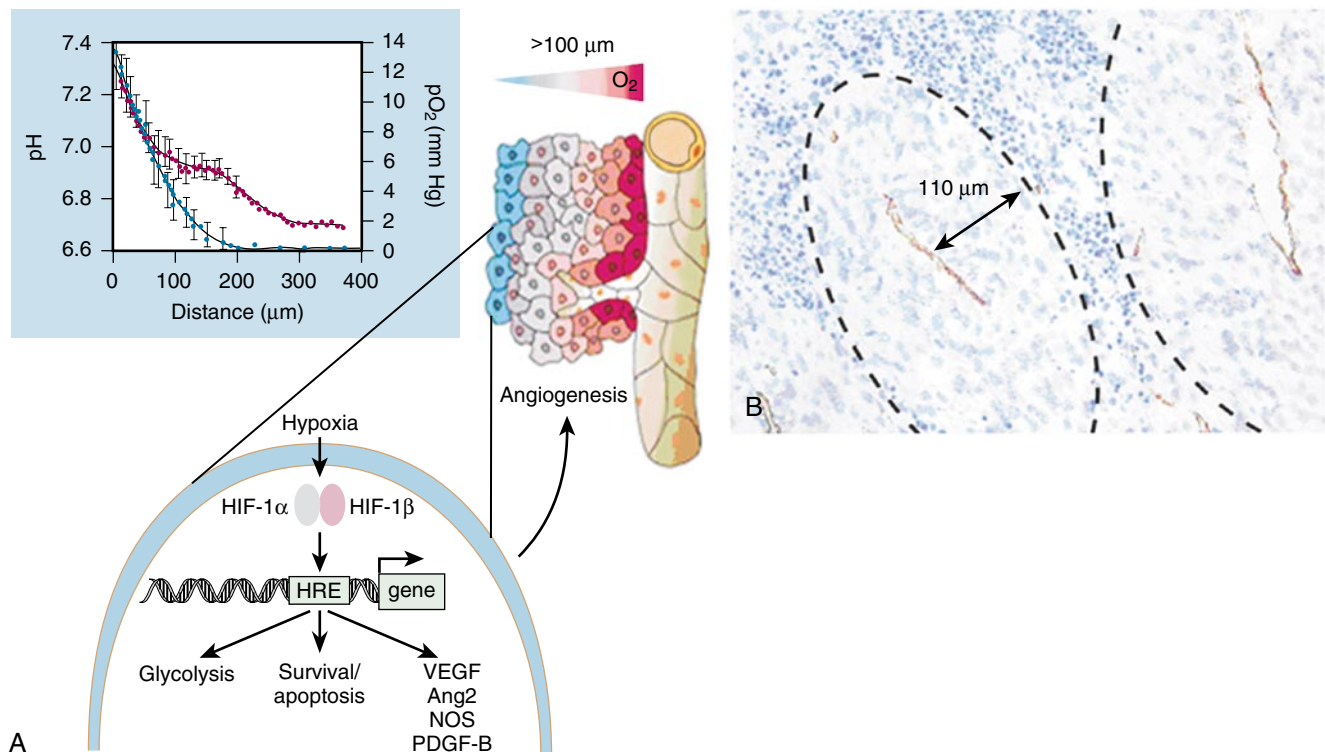


FIGURE 18-4 A: Because of the irregular pattern and organization of the tumor vasculature, some cells in tumors are located more than 100 μm (the diffusion limit for oxygen) away from blood vessels and become hypoxic (red-to-blue gradient indicates progressive hypoxia). Tumor cells survive fluctuations in oxygen tensions, in part because clones are selected in hypoxic tumors that switch to a pro-angiogenic phenotype. Hypoxia-inducible transcription factors (HIFs) increase transcription of several angiogenic genes (for example, genes encoding vascular endothelial growth factor [VEGF], platelet-derived growth factor [PDGF-BB], and nitric oxide synthase [NOS]). HIFs also affect cellular survival/apoptosis pathways. **Inset:** Relationship between the distance of tumor cells from nearby vessels and their degree of hypoxia (blue symbols) and acidosis (red symbols). (From Carmeliet P, Jain RK. *Angiogenesis in cancer and other diseases*. *Nature* 2000;407:249–257, with permission.) **B:** Section of rat prostatic carcinoma in which vessels were identified by CD31 immunostaining. A “cuff” of viable cells surrounds each capillary, beyond which regions of necrosis are evident. (From Hltaky L, et al. *J Natl Cancer Inst* 2002;94:883–893, by permission of Oxford University Press.)

Research data, however, suggest that heterodimers of PlGF and VEGF may be more potent in some contexts than the more typical VEGF homodimer (24). Much work remains to be done to tease apart the unique and overlapping functions of the various VEGF homologues and their receptors.

Basic Fibroblast Growth Factor

Basic FGF (bFGF or FGF2) is one of 22 known fibroblast growth factors that mediate a large number of developmental and homeostatic functions in different tissues. bFGF was identified biochemically in a search for angiogenic molecules released by tumor cells. When added to tissues exogenously or overexpressed in transplanted tumor cells, bFGF has potent angiogenic properties (47). Like VEGF, bFGF binds to heparin sulfate proteoglycans and activates cognate receptor tyrosine kinases. Interestingly, loss-of-function studies have failed to reveal an inherent role of bFGF in embryonic angiogenesis, although this may be due to functional complementation by other FGF family members. As many of the angiogenic properties of bFGF appear to require VEGF function, however, one important role of bFGF in tumor angiogenesis may be to induce VEGF expression (47). The situation is almost certainly more complex, as VEGF and bFGF act synergistically in some contexts, but clearly have independent effects on ECs in others. For example, bFGF (but not VEGF) induces telomerase expression in ECs, possibly inhibiting cell senescence, whereas

VEGF (but not bFGF) confers changes in EC fenestration (47). The emerging picture would suggest that bFGF and many other angiogenic factors act as general growth and survival factors for ECs partly by regulating VEGF expression, whereas VEGF itself may preferentially stimulate many of the cellular processes that lead to new vessel formation.

Angiopoietins/Tie Receptors

In addition to VEGF and FGF receptors, ECs express the Tie1 and Tie2/Tek receptor tyrosine kinases. Genetic ablation of Tie1 or Tie2 in mice produced embryos in which vasculogenesis was intact, but subsequent angiogenic remodeling was inhibited. Soluble forms of these receptors were used to identify endogenous ligands, called angiopoietins (Ang1–Ang4) (48,49). Deletion of Ang1 produced a phenotype similar to loss of Tie2, supporting a role for Ang1 as an important activator of Tie2 signaling. Interestingly, Ang2 also binds to Tie2 with high affinity, but does not stimulate Tie2 tyrosine phosphorylation or downstream signaling. Transgenic overexpression of Ang2 produced a phenotype similar to that associated with loss of Ang1 or Tie2, suggesting that Ang2 may be a naturally occurring inhibitor of Ang1 signaling. The role of Ang2 became clearer when it was shown to be induced in concert with VEGF at sites of vascular remodeling. Several studies have suggested a model in which Ang2 interferes with the stabilizing effects of Ang1 (such as increased pericyte and smooth muscle recruitment)

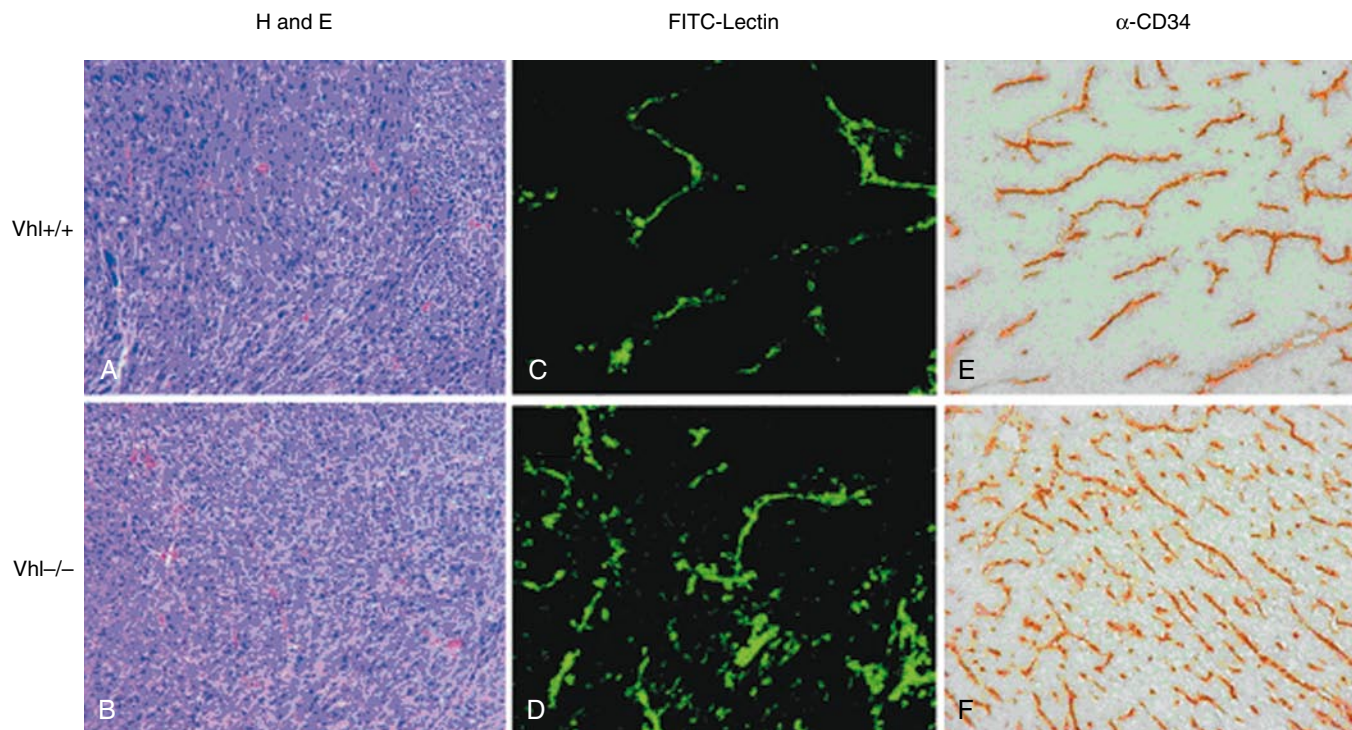


FIGURE 18-5 LOSS OF THE pVHL TUMOR SUPPRESSOR INCREASES TUMOR ANGIOGENESIS. Fibrosarcomas were generated subcutaneously in immunocompromised mice by injecting Ras-transformed fibroblasts derived from wild-type (*Vhl*^{+/+}) or pVHL-deficient (*Vhl*^{-/-}) mice. Tumor sections reveal that loss of pVHL, and consequent constitutive hypoxia-inducible transcription factor (HIF) activation, correlated with increased tumor angiogenesis. Tumor vessels were labeled with either FITC-lectin (**B, E**) or CD34 antibodies (**C, F**). H&E, hematoxylin and eosin (**A, D**). (Courtesy of F. Mack and M. C. Simon.)

thereby allowing VEGF to stimulate EC division and migration more efficiently (49). The roles of Ang3 and Ang4 are less clear, and a cognate ligand for Tie1 has not been identified (48).

Platelet-Derived Growth Factor

Maturation and maintenance of the vascular system requires the establishment of a close functional relationship between ECs and pericytes (PCs). ECs undergoing active division and morphogenesis express PDGF-B at the apical end of their cell surface, and PCs express the corresponding receptor PDGFR β . Genetic ablation of ligand or receptor in mice disrupts PC recruitment, resulting in leaky, malformed blood vessels and increased EC apoptosis (50). Bergers and colleagues identified a population of c-Kit⁺, Sca-1⁺ bone marrow progenitor cells that are recruited to perivascular sites in tumors, where they differentiate into PCs and stabilize the tumor vessels in a PDGFR β -dependent manner (51). Overexpression of PDGF promoted recruitment of PCs and tumor vessel stabilization, whereas inhibition of PDGF signaling reduced PC recruitment with a concomitant increase in EC apoptosis (32). Consequently, a combination of therapies that target both tumor ECs and PCs may prove to be a particularly effective approach (32,52).

Anti-Angiogenic Factors

In his landmark 1971 paper, Judah Folkman (1) not only proposed that tumor growth depends on angiogenesis, but also suggested that angiogenic inhibitors could be identified and used

therapeutically. Intensive efforts over the subsequent three decades have led to the identification of at least 27 endogenous inhibitors whose application can block angiogenesis in a variety of assays and genetic models (6,53). Some of these naturally occurring compounds (thrombospondin, endostatin, and tumstatin) are proteolytic cleavage products of extracellular matrix proteins. Other endogenous inhibitors include interferons, interleukins, proteolytic fragments of the protease plasminogen (angiostatin), and clotting factors (cleaved antithrombin III and prothrombin kringle-2) (53). The specific functions of these compounds in tumor angiogenesis and their possible utility as therapies for cancer treatment is under active investigation.

Thrombospondin-1

Initially identified as an extracellular glycoprotein with cell adhesive properties, thrombospondin-1 (TSP-1) binds to integrin and nonintegrin cellular receptors, cytokines, growth factors, and extracellular proteases. TSP-1 is thought to act as a molecular scaffold that facilitates interactions between factors controlling cell morphology, signaling, and adhesion, possibly by promoting receptor clustering (54). In 1990, Bouck, Polverini, and colleagues described the strong anti-angiogenic activity of a TSP-1 proteolytic fragment (55). Targeted deletion of TSP-1 in mice increased tumor angiogenesis and growth, and subsequent reports confirm the inability of TSP-1 mutant mice to mount a normal angiogenic response in other assays (56). The TSP-1 gene has been shown to be a direct target of the p53 tumor suppressor, and TSP-1 expression has been inversely correlated with the progression of carcinomas and

melanoma in humans (53). The molecular mechanisms by which TSP-1 blocks angiogenesis are likely to be complex, but may include integrin inhibition, interference with VEGF and bFGF signaling, and/or induced expression of the pro-apoptotic FasL protein on ECs (53). The identification of TSP-1 as a direct p53 target suggests yet another mechanism whereby p53 inactivation can promote tumor progression.

Endostatin and Tumstatin

Both endostatin and tumstatin are proteolytic cleavage fragments derived from collagen molecules. Endostatin was initially purified from a murine hemangioendothelioma cell line and identified as a 20-kD carboxy-terminal fragment of type XVIII collagen. Recombinant endostatin has multiple anti-angiogenic properties, including the ability to interfere with VEGF and bFGF signaling, inhibit EC motility, and induce EC cell cycle arrest and apoptosis (53). Endostatin appears to mediate these pleiotropic effects by binding EC integrins, including $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_1$. Tumstatin consists of a 28-kD fragment of the $\alpha 3$ chain of type IV collagen, promotes EC apoptosis, and suppresses the growth of various human tumor cells in xenograft experiments. Similar to endostatin, tumstatin binds to integrins and thereby inhibits activation of downstream signaling pathways (6,53). Despite their similarities, endostatin and tumstatin peptides share little sequence identity and can clearly mediate independent functions: For example, endostatin inhibits EC migration with little effect on VEGF-induced proliferation, whereas tumstatin inhibits EC proliferation without significantly affecting migration. These functional differences may be explained by the finding that endostatin preferentially inhibits the FAK/c-Raf/MAPK-ERK1,2/p38/MAPK1 signaling pathway, whereas tumstatin inhibits the FAK/PI3K/Akt/mTOR/4E-BP1 pathway that controls cap-dependent protein translation (53).

It is interesting to note that many endogenous angiogenesis inhibitors are generated by proteolytic degradation of ECM proteins, or from proteins involved in blood clotting, and that many bind directly to integrin receptors. Growing evidence supports the notion that these compounds play an important role in fine-tuning the angiogenic response that accompanies thrombosis and tissue repair (30). Consequently, their activity in limiting tumor angiogenesis may reflect the idea that the tumor microenvironment, with infiltrating inflammatory cells and activated fibroblasts, is thought to resemble a “wound that never heals” (20). The production of these endogenous angiogenesis inhibitors may also help explain tumor dormancy, as first proposed by Folkman in 1971. If local angiogenic activity in a tumor is controlled by the balance of pro-angiogenic factors (VEGF, angiopoietin 1, bFGF, etc.) and angiogenesis inhibitors (TSP-1, endostatin, tumstatin, etc.), then it may take months or years to generate the proper genetic and physiologic conditions necessary to tip the balance—or throw the **angiogenic switch**—to favor active blood vessel development and tumor growth. Multiple genetic models in mice have shown that tumors generated by transgenic expression of oncogenes initially remain small, with tumor cell proliferation largely offset by apoptosis (7). After a period of relative stasis, the tumors begin to show evidence of increased vascularity after which they grow rapidly (7), consistent with activation of the angiogenic

switch. The synthesis of angiogenesis inhibitors by a primary tumor may also keep distant metastases from progressing, as removal of a large primary tumor often correlates with the rapid outgrowth of previously unidentified metastatic tumors in patients (1,6).

Targeting Tumor Angiogenesis in Patients

The ever-increasing list of factors regulating the angiogenic switch in tumors provides opportunities for new clinical therapies. In addition to drugs designed to target specific molecules, a number of other drugs (including thalidomide, interferon- β , fungal metabolites [fumagillin], and receptor tyrosine kinase inhibitors [RTKIs]) have been shown to inhibit angiogenesis in preclinical models and to affect tumor growth in clinical trials. Results, however, have been mixed: Early trials of endostatin and other compounds that showed promise in preclinical models yielded disappointing results in the clinic (57,58). In contrast, a more recent trial showed a positive effect of angiostatin in treating non-small cell lung cancer when combined with cytotoxic chemotherapy (59). Some reasons for the apparent discrepancies between preclinical results and patient responses are discussed in the final section of this chapter. Nevertheless, the success of targeted anti-angiogenic therapies suggests that this will be an increasingly important strategy for treating cancer and other vascular diseases in the years to come.

The dependence of tumor vascularization on VEGF makes it an obvious therapeutic target. In 1993, Ferrara and colleagues reported that a murine antihuman VEGF monoclonal antibody could inhibit the growth of different human tumor cell lines in immunocompromised mice, although the antibody had no effect on tumor cell proliferation in vitro (60). Subsequent analysis revealed that the antibody blocked angiogenic activity in these xenografts and led to the development of a “humanized” version of the antibody, called bevacizumab or Avastin, for human clinical trials. In 2003, results from two clinical trials of Avastin function generated tremendous excitement in the field. In one phase 3 trial, patients with advanced metastatic colorectal cancer were treated with Avastin in conjunction with cytotoxic chemotherapy (61) and displayed an average increase in survival of approximately 4 months (from 16 to 20 months). Although this response seems modest, it was the first indication that specific targeting of VEGF in highly metastatic human cancer could have a survival benefit. In a separate phase 2 trial, patients with metastatic renal cancer showed a significant, dose-dependent increase in time-to-progression when treated with Avastin compared with placebo (62).

VEGF signaling can also be inhibited by other therapeutic compounds. Pegaptinib, an aptamer that inactivates VEGF-165, is used for treatment of the “wet” or neovascular form of age-related macular degeneration, underscoring the utility of targeting VEGF in multiple vascular diseases (21). Another example is TNP-470, a synthetic version of the fungal compound fumagillin that inhibited tumor growth in clinical trials (6). Toxicity problems prompted development of caplostatin, a less-toxic version of TNP-470, which appears to function at least in part by interfering with VEGFR-2/Flk-1 receptor phosphorylation and signaling (9).

A number of chemotherapeutic drugs initially characterized as immunomodulators have also been shown to affect tumor vascular development, either directly or indirectly. Interferon- α , identified as an angiogenesis inhibitor in the 1980s, has been used successfully to treat hemangiomas, angioblastomas, and other cancers, apparently by blocking bFGF expression (9). Another example is thalidomide, a sedative (and teratogen) later shown to alter interleukin synthesis and T-cell function, which inhibits angiogenesis *in vitro*. Thalidomide is now used in the treatment of multiple myeloma (9), although its pleiotropic effects on the immune system make it difficult to assess the degree to which its clinical effectiveness depends on blocking angiogenesis *per se*.

Efforts to design or identify molecules that inhibit disparate intracellular signaling transduction pathways have also uncovered unexpected effects on tumor angiogenesis. Tarceva inhibits epidermal growth factor (EGF) receptor tyrosine kinase activity, and consequently reduces VEGF expression. In addition, several small-molecule kinase inhibitors with broad target specificity can inhibit VEGF and/or PDGF receptors, thereby inhibiting angiogenic signaling. Sorafenib (Bayer-43-0009, Nexavar) is a Raf kinase inhibitor in clinical trials for treatment of melanoma and other malignancies. Interestingly, sorafenib also inhibits VEGFR-2/Flk-1, VEGFR-3/Flt-3, and PDGFR β and may provide an anti-angiogenic benefit. Sorafenib and another small molecule, sunitinib (Sugen11248), which inhibits c-Kit, all three VEGF receptors, and PDGFR β , are used in the treatment of kidney cancer.

In considering the apparently modest effects of Avastin treatment on cancer patient survival and time to progression, it is worth noting that the patients enrolled in the trials typically had advanced, metastatic disease that was resistant to standard therapies. Consequently, it will be important to determine the effects of Avastin, or any other anti-angiogenic therapy, when used to treat patients early in the course of their disease. The most effective time to use anti-angiogenic therapies may be before tumors become detectable by physical examination or standard imaging—namely, before the angiogenic switch has been thrown. This strategy will depend on the identification of reliable biomarkers that indicate early stages of tumorigenesis (6,66).

Anti-angiogenic drugs appear to be most effective in treating cancer patients when delivered in combination with standard cytotoxic chemotherapeutics. This is somewhat counterintuitive: If a functional vascular system is necessary to deliver cytotoxic drugs designed to kill rapidly dividing tumor cells, then how can inhibition of vascular development increase their efficacy? Several models explain this observation, none of which is mutually exclusive with the others. First, if angiogenic inhibitors reduce the overexpression of VEGF and other compounds, it may restore a normal balance of pro- and anti-angiogenic factors, thereby establishing a more “normalized” vascular system. This, in turn, could allow more uniform delivery of the cytotoxic drugs, reduce interstitial tumor pressure and vessel leakage, and reduce hypoxia, all of which could inhibit tumor growth. Verification of this intriguing idea, proposed by Jain and colleagues (22), will require additional investigation and clinical testing.

Metronomic or “Low-Dose” Therapy

A different model suggests that combining anti-angiogenic and cytotoxic chemotherapies is effective because the former may preferentially target ECs, whereas the latter targets circulating endothelial precursors (CEPs). In the clinic, treatment with drugs at the MTD (maximum tolerated dose) is typically followed by a “break” period to allow the patient to recover from the toxic and myelosuppressive effects of the treatment. MTD chemotherapy typically causes a significant decrease in the number of circulating hematopoietic cells, including neutrophils and other myeloid cells, as well as CEPs. These drops can be quite precipitous, and are usually followed by a rapid “rebound” period in which circulating progenitors are mobilized from the bone marrow, a response which has been observed both in mice and humans. One potentially unfortunate consequence of this response is the increase in CEPs, which appear capable of differentiating into mature endothelial cells. The additional recruitment of bone marrow–derived myeloid cells, such as Tie-2–expressing monocytes, could contribute directly or indirectly to tumor angiogenesis (63). The “breaks” in MTD regimens may therefore allow repair or expansion of the tumor vasculature and thereby reduce cytotoxic benefit. Although the precise nature and function of CEPs and their differentiated progeny cells remain controversial, there is evidence that VEGF and other angiogenic factors stimulate their release from the bone marrow (26). Therefore, the addition of anti-angiogenic drugs to standard chemotherapeutic treatments may suppress the ability of tumors to recruit CEPs and their progeny during the drug-free break periods between MTD treatments.

The reduction in drug-free breaks appears to have an additional inhibitory effect on tumor angiogenesis. In 2000, the effects on tumor growth in mice was greater when an MTD regimen was changed to one in which animals were treated with low doses of the same drug, but at more frequent intervals (65). Surprisingly, tumor growth was inhibited or reversed, despite the fact that (in some cases) the tumor cells were themselves resistant to the same cytotoxic drug! These results suggest that the chemotherapy was not only targeting the tumor cells, but also inhibiting normal cells such as ECs or recruitment of CEPs. Subsequent work showed that regular, low-dose chemotherapy (also termed “metronomic” dosing) induced the expression of the angiogenic inhibitor TSP-1 in mice and that genetic deletion of TSP-1 promoted tumor growth and angiogenesis in this model (56). In fact, one might predict that normal ECs, which are neither transformed nor genetically unstable, would be more sensitive to the cytotoxic effects of chemotherapeutics than tumor cells. By treating patients with a sustained, low dose of the drug and avoiding the breaks inherent to MTD regimens, it is possible that EC recruitment to the tumor vasculature could be more uniformly suppressed, thereby limiting tumor growth. By incorporating targeted anti-angiogenesis drugs such as Avastin, it may also be possible to use a sufficiently low dose of cytotoxic agents to inhibit EC proliferation, survival, and function, without severe myelosuppressive effects. The study of metronomic therapy is in its early days, but there are some compelling clinical precedents in which patients with non–small cell lung, metastatic breast, or ovarian cancers responded to treatments of the DNA-damaging

drug etoposide or microtubule-inhibiting taxanes delivered weekly at reduced levels compared with MTD (67). The lower doses of these drugs would also reduce toxic side effects, alopecia, nausea, and so forth and thereby greatly improve the patient's quality of life. The encouraging preclinical and clinical results have prompted the establishment of ongoing metronomic chemotherapy clinical trials focusing on breast, prostate, lung, and pancreatic cancers, as well as melanoma, hepatocellular carcinoma, and others (67).

Remaining Challenges

The preliminary success of Avastin and other anti-angiogenic therapies suggests that oncologists will have a new and growing arsenal of weapons to complement standard chemo- and radiation-based therapies in the future. As always, caution is necessary, as preclinical data rarely predict the exact outcome of treatments in patients. One reason for this discrepancy is that many preclinical studies have continued to rely on xenograft models, in which a large number of highly malignant tumor cells are introduced subcutaneously into recipient mice. Although a quick and reproducible approach, it is perhaps not surprising that events that are rate-limiting for xenograft growth may have little to do with those

controlling human cancer progression. Some anti-angiogenic compounds, such as endostatin, profoundly limited or regressed tumor growth in xenografts but failed to show any significant benefit in early clinical trials (57,58). The development of genetically altered strains of mice that more closely mimic the development and histology of human cancers (68) may offer more predictive preclinical models for anti-angiogenic therapy.

It is increasingly clear that anti-angiogenic therapies are likely to be most effective when combined with other treatments, for the reasons elaborated in the preceding sections. At this stage, it is essentially impossible to predict which specific combinations of drugs, and which specific delivery strategies, are likely to be effective in inhibiting angiogenesis for a given tumor type or in a given patient (66). Finally, it is almost certain that tumors will eventually develop resistance to specific angiogenesis inhibitors, either by modulating the balance of pro- and anti-angiogenic factors or by acquiring additional genetic changes. A great deal more research will be necessary to establish even the most general guidelines, but the potential benefits of treating cancer patients with angiogenesis inhibitors, particularly early in the progression of disease, are considerable. It is likely that our understanding of tumor angiogenesis, and our ability to manipulate it clinically, will have altered greatly by the next edition of this book.

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Invasion and Metastasis

In the written history of medicine, neoplasms have been diagnosed for nearly 4,000 years. Almost from the beginning, medical practitioners recognized that the most life-threatening attribute of neoplastic cells is the ability to disseminate and colonize distant tissues. When tumors are diagnosed and have not spread beyond the tissue of origin, cure rates for most cancers approach 100%. However, when tumor cells have established colonies elsewhere, cancer is often incurable.

The process of converting a normal cell into a life-threatening metastatic cancer cell is referred to as tumor progression (Figure 19-1). As discussed in previous chapters, medicine has evolved toward a recognition that neoplasia is a cellular disease, and further advanced to understand the molecular underpinnings of the early stages of progression resulting in cancer development. It is now recognized that metastases represent a subset of cells that have left the primary tumor, which are behaviorally distinct from the cells remaining at the site of tumor origin, and the molecular mechanisms underlying the phenotypic differences that characterize a metastatic cell are being elucidated.

Generation of a Metastatic Cell

Metastasis is defined as the dissemination of neoplastic cells to discontinuous nearby or distant secondary sites where they proliferate to form a mass. But how did tumor cells acquire the ability to metastasize? The answer to this question requires examination of the mechanisms underlying how tumors arose and progressed toward increasingly aggressive behavior.

By the time a neoplasm is diagnosed, it comprises at least 10^9 cells. Yet, even cursory examination of a tumor histologically reveals that the cells are pleomorphic. Furthermore, if one isolates single cell clones from a tumor, they vary dramatically in terms of biological behavior.

Tumor heterogeneity exists for virtually every phenotype measured (1,2). There are three types of heterogeneity within a tumor: positional, temporal, and genetic. Positional heterogeneity is determined by the accessibility of a cell to external stimuli (e.g., oxygen [O_2] levels). For example, radiation sensitivity is proportional to oxygenation; therefore, two identical cells would exhibit differences in radioresponse depending on distance from a capillary. Temporal heterogeneity is relevant with regard to changes in

cells due to cyclical signals. Cells in the G_0/G_1 phase of the cell cycle would be less sensitive than cells in S phase to drugs targeting DNA replication. Genetic heterogeneity is the result of inherent properties of tumor cells themselves. Isolation of single-cell clones confirms that there are inherent differences between subpopulations comprising a single tumor mass.

The heterogeneity of tumors raises an important question regarding tumor origin: Are tumors of unicellular or multicellular origin? Tumors express maternal or paternal isoenzymes, but rarely both, strongly suggesting that they arose from a single cell. Analysis of karyotypes reveals that virtually all cells within a tumor share a common abnormal chromosomal change [e.g., all CML cells have t(9;22)]. Additional karyotypic abnormalities may be superimposed on the shared ones. If tumors are monoclonal, how, then, does heterogeneity arise?

Generation of heterogeneity requires divergence of single transformed cells into multiple phenotypically distinct progeny. The process appears to be fundamental to tumor progression, but also occurs in normal physiology. For example, pluripotent hematopoietic stem cells can generate cells along multiple lineages, and a single fertilized egg yields a multicellular organism with organs and tissues. While stem cell theory accommodates diversification, the molecular mechanisms underlying differentiation and diversification of both normal and cancer cells are still being elucidated (see Chapter 10).

One of the first formalized conceptual frameworks of tumor progression was introduced by Peyton Rous, who described the steps involved in the transformation of skin and breast carcinomas (3).

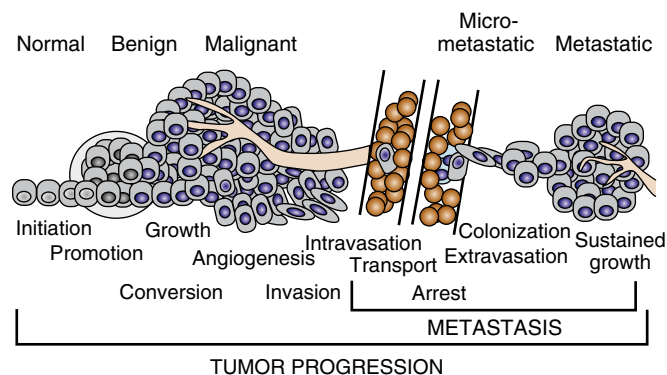


FIGURE 19-1 TUMOR PROGRESSION.

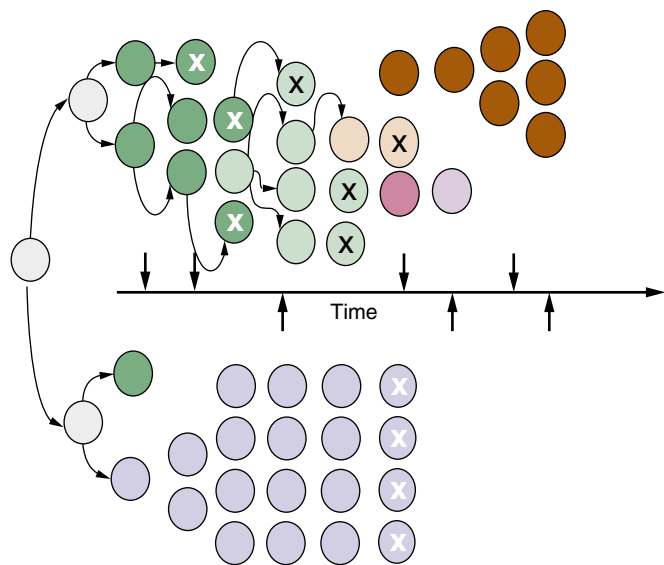


FIGURE 19-2 MUTATION-SELECTION THEORY OF TUMOR PROGRESSION. In general, tumor cells have higher rates of mutation than normal cells; however, the mutation rates vary by cell. With low mutation rates, the population is more susceptible to a lethal selective pressure (*blue cells*). The upper series of cells are generating variants continually, some of which are eliminated by selective pressures (*arrows*) or some of which are overwhelmed by other cells with more robust growth characteristics (*green with X*). Note that the cells comprising the population are different over time. The change in population composition is the basis of tumor progression.

His concepts were expanded by Leslie Foulds, who studied the acquisition of hormone independence by mammary tumors. Foulds defined progression as “the acquisition of permanent, irreversible qualitative changes of one or more characteristics in a neoplasm” (4,5). Rous and Foulds provided evidence that tumor progression occurs in a constant, unique, and stepwise pattern. The trend is toward increased autonomy; however, individual characteristics within a tumor independently assort.

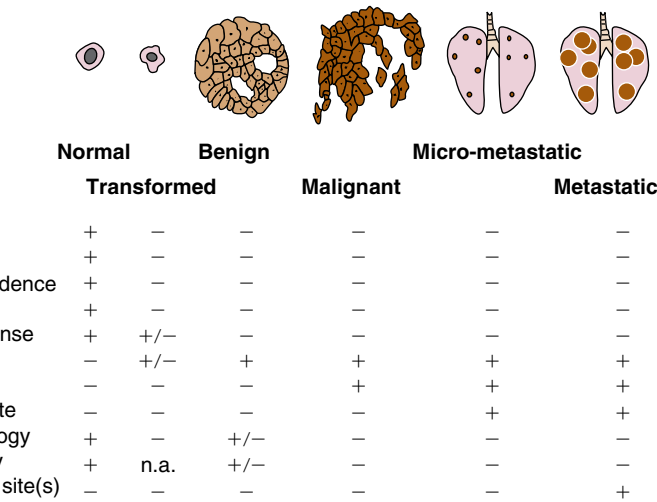
The mutation-selection theory of tumor progression proposes that genetic instability within a tumor provides for the random generation of variants within the population (Figure 19-2). Expanding on Boveri’s original observations that alterations of chromosomal material were significant in the generation and progression of tumors,

Nowell proposed that neoplastic cells are more genetically unstable than normal counterparts (6). Fluctuation analyses for a variety of genes and phenotypes show that transformed cells are significantly (frequently 10,000- to 100,000-fold, but as high as 10⁷-fold higher) more genetically unstable than normal counterparts. If mutations are coupled with selective pressures, tumor progression would occur as a result of mutation and coupled selection. Epigenetic modifications on the cells and selection pressures imposed by the host and/or competition with other cells alter tumor composition via Darwinian selection principles. Subpopulations of cells within the original tumor will acquire the ability to migrate and establish themselves at other sites. This capacity would offer a selective advantage since tumor cells will not be limited by space or location.

Isaiah Fidler and Margaret Kripke tested these hypotheses with regard to the metastatic phenotype using combinations of cloning and Luria-Delbruck fluctuation analysis (7). Single-cell clones isolated from a single tumor varied considerably in their metastatic potentials. Poste and colleagues later showed that highly metastatic cells, when grown in culture continuously and recloned, yielded populations that contained non- or poorly metastatic cells (8). Likewise, continuous culture of poorly metastatic cells yielded subpopulations that were highly metastatic. In other words, the clonal populations did not remain homogeneous.

At the cellular level, tumor progression typically follows a sequence as depicted in Figure 19-3. Before becoming tumorigenic, cells lose the ability to differentiate fully, are no longer contact inhibited or anchorage dependent, and have acquired genetic instability. The ability to form a neoplastic mass (i.e., tumorigenicity) typically goes through a phase with expansile growth in the absence of invasion. Although cells may be pleomorphic at this stage, they are often encapsulated by a dense fibrous capsule. Tumors that have failed to invade through a basement membrane are referred to as benign or carcinoma in situ. With continued generation of variants and selection, subsets of the cells acquire the ability to escape through a basement membrane, the hallmark of malignancy. Acquisition of the abilities to detach from the primary tumor and move elsewhere are required for metastasis. It is important to clarify that tumor progression is typically measured in terms of the tumor mass, rather than by the individual cells within it. The stage of a tumor

FIGURE 19-3 PROPERTIES OF CELLS IN THE TUMOR PROGRESSION CONTINUUM. The indicated cells along the tumor progression continuum either display (+), do not display (–), or sometimes display (+/–) the indicated phenotypes. n.a., not applicable.



can be defined by the most malignant cells found. Even if more than 99% of cells are indolent, a tumor is defined as malignant if a single cell has penetrated a basement membrane.

At the molecular level, certain chromosomal and genetic changes are more prevalent in early versus late stages of tumor progression (see Chapter 1), despite unpredictability in specific genetic changes occurring within a cell. Use of this information has allowed prediction of genetic underpinnings controlling tumorigenesis, invasiveness, and metastasis.

Tumor Invasion

Tumor invasion, the capacity for tumor cells to disrupt the basement membrane and penetrate underlying stroma, is the distinguishing feature of malignancy. Invasion requires major changes in cell morphology and phenotype, in particular for epithelial cells that represent the precursors to over 90% of human cancers. Normal epithelial cells form polarized sheets maintained by tight junctions, adherens junctions that organize the actin (microfilament) and tubulin (microtubule) cytoskeleton, and desmosomes attached to keratin-containing intermediate filaments. They are anchored to the basement membrane by hemidesmosomes and their associated intermediate filaments and integrin contacts that organize actin. Invasion requires alterations in cell–cell and cell–matrix adhesion, coordinated with matrix degradation and cellular motility (Figure 19-4; 9). The structural and regulatory proteins that control cell adhesion and migration are key downstream targets of oncogene and tumor suppressor–controlled signaling pathways, providing insights into how oncogenic transformation results in progression to an invasive phenotype. An interesting observation has been that many of the molecules implicated in tumor invasion also affect other processes involved in tumor progression, including cell survival, growth, apoptosis, and angiogenesis, highlighting the intricacy of the network of interrelated pathways that controls cellular behavior (10).

Adhesion

Invasion of epithelial cell–derived carcinomas often involves dramatic changes in cell shape. Conversion from an epithelial morphology to a nonpolarized, motile, spindle-shaped cell resembling a fibroblast is referred to as the epithelial–mesenchymal transition (EMT; 11). EMT is characterized by the loss of epithelial-specific

E-cadherin from the adherens junctions and a switch from the expression of keratins as the major intermediate filament to the mesenchymal intermediate-filament vimentin. EMT is not cancer-specific; it is a normal process that occurs during embryonic development and wound healing. EMT is influenced by the tumor microenvironment and is observed primarily at the edge of the tumor in contact with tumor stroma. Soluble factors, in particular transforming growth factor- β and hepatocyte growth factor/scatter factor, are regulators of EMT. Tumor cells may reverse the process and undergo a mesenchymal–epithelial transition (MET) in the absence of EMT-inducing signals. The transient nature of EMT helps explain why metastatic cells can morphologically resemble cells in the primary tumor despite the fact that they by necessity have accomplished all the steps of the metastatic cascade.

Epithelial cell–cell interactions are mediated primarily by cadherins, transmembrane glycoproteins that form calcium-dependent homotypic complexes. The epithelial-specific cadherin, E-cadherin, functions as a tumor suppressor and a metastasis suppressor (12). Loss of E-cadherin correlates with increased invasion and metastatic potential in most tumor types. Reexpression of E-cadherin in experimental models can block invasion, suggesting that E-cadherin loss is indeed causative. Loss of E-cadherin in cancer occurs through several mechanisms, including transcriptional repression and proteolytic degradation. The zinc finger transcriptional repressors Snail and Slug, in particular, have been implicated in regulating EMT by virtue of their ability to repress E-cadherin transcription. Cadherins are regulated by catenins (α -, β -, γ -, and p120 catenins), cytoplasmic proteins that functionally link the cadherin complex to the actin cytoskeleton. β -catenin is a cell adhesion protein and a transcription factor. In addition to its role in adherens junctions, it participates in canonical Wnt signaling, a signaling pathway important in development and cancer (see Chapter 11). E-cadherin levels and function are also disrupted by loss of p120 catenin, which occurs in many tumor types and may also contribute to tumor metastasis.

Loss of function of cell–cell adhesion molecules other than E-cadherin is associated with the ability of tumor cells to invade and metastasize. Neural cell adhesion molecule (NCAM), a member of the immunoglobulin-like cell adhesion molecule Ig-CAM family, is down-regulated in several tumor types, and NCAM loss results in an increased ability of tumor cells to disseminate (12). Other Ig-CAMs, such as DCC (deleted in colorectal carcinoma), CEACAM1 (carcinoembryonic antigen CAM1), and Mel-CAM (melanoma-CAM) also demonstrate reduced expression in specific cancer types. However, not all cell–cell adhesion molecules

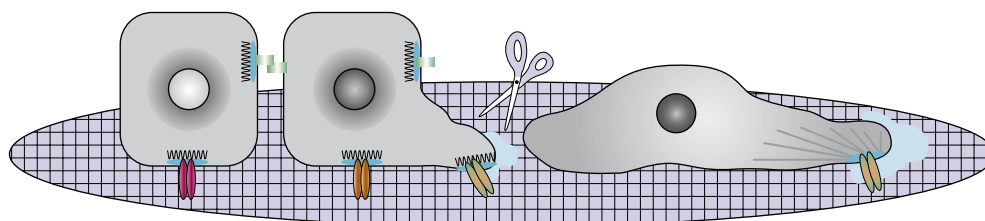


FIGURE 19-4 THE STEPS OF TUMOR INVASION. Tumor invasion involves the loss of cell–cell adhesions (cadherins represented by green bars), alterations in cell–matrix adhesion (integrins represented by ovals), proteolysis of the extracellular matrix (blue matrix, degradation demonstrated by clearing of matrix mediated by proteinases represented by scissors) and motility involving alterations in the actin cytoskeleton (intracellular black and gray lines).

can be viewed as potential invasion suppressors. N-cadherin promotes motility in some cell types, and Ig-CAMs such as L1, CEA (carcinoembryonic antigen), and ALCAM (activated leukocyte CAM) are often overexpressed in advanced cancers and have functions associated with cancer progression. This complexity may be explained by signaling functions for these molecules, either direct or indirect, that are distinct from their role in cell–cell adhesion. The interrelatedness of tumor growth and tumor invasion, and limitations of experimental model systems, often does not allow a distinction between growth effects that influence the appearance of an invasive phenotype and an effect on cellular invasion per se.

The ECM provides a scaffold for the organization of cells and spatial cues that dictate cell behavior (13). The extracellular matrix is composed of proteins, primarily triple-helical collagens, glycoproteins such as laminins and fibronectin, and proteoglycans. The basement membrane is an organized ECM that separates polarized epithelial, endothelial, and muscle cells from the underlying tissue. Interstitial matrix provides the structure characteristic of connective tissues. The molecular composition of the ECM varies between tissues and organs, and provides contextual information to cellular constituents. In addition, the ECM serves as a repository for secreted regulatory proteins and growth factors. Finally, ECM proteins themselves can be active signaling molecules, activities that frequently are only revealed after proteolysis reveals cryptic sites. Thus, the interaction of cells with ECM molecules determines their capacity for survival, growth, differentiation, and migration.

Cells adhere to ECM via integrins, a family of transmembrane glycoproteins assembled as specific combinations of 18 α and eight β subunits (14). Integrins bind to distinct but overlapping subsets of ECM components. During tumor progression, cancer cells tend to undergo a switch in their integrin expression pattern, down-regulating the integrins that mediate adhesion and maintain a quiescent, differentiated state, and expressing integrins that promotes survival, migration, and proliferation (15). Although there is a cell-type dependency on integrin function, in general integrins $\alpha_5\beta_1$ and $\alpha_3\beta_1$ are viewed as suppressors of tumor progression, while $\alpha_5\beta_3$, $\alpha_v\beta_3$, and $\alpha_6\beta_4$ promote cellular proliferation and migration. Integrins mediate both “outside-in” and “inside-out” signaling, so that changes in cellular adhesion can alter cellular phenotype, and changes in intracellular signaling pathways can modulate cellular adhesion. A well-described and important mechanism whereby integrin–ECM interactions modulate cell function is by cooperative signaling with different growth factor receptors. Many of the cellular responses induced by activation of tyrosine kinase growth factor receptors are dependent on the cells being able to adhere to an ECM substrate in an integrin-dependent fashion. Signaling in response to ECM ligation usually activates focal adhesion kinase (FAK) and nonreceptor tyrosine kinases of the *src* family.

Matrix Degradation

Disruption of basement membrane is a hallmark of malignancy. Degradative enzymes produced by the tumor cells, and by resident and infiltrating cells as a response to the tumor, contribute to

matrix degradation and facilitate tumor cell invasion. Proteolytic enzymes of many classes have been implicated in tumor cell invasion, including the serine proteinases plasmin, plasminogen activator, seprase, hepsin, several kallikreins, the cysteine proteinase cathepsin-B, the aspartyl proteinase cathepsin-D, and metal-dependent proteinases of the matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase (ADAM) families. Other matrix-degrading enzymes such as heparanase, which cleaves heparin sulfate proteoglycans, and hyaluronidase cleavage of its substrate hyaluronic acid have also been causally associated with tumor progression and invasion.

Liotta and colleagues observed that metastatic potential correlates with the degradation of type IV basement membrane collagen and focused attention on the metal-dependent gelatinases (16). These enzymes are now recognized as MMP2 and MMP9, and many of the 23 members of the MMP family of matrix-degrading metalloproteinases have been associated with tumor progression. Elevated MMP levels correlate with invasion, metastasis, and poor prognosis in many cancer types, and animal models provide evidence for a causal role for MMP activity in cancer progression (17). The plasminogen activator/plasmin system has also been causally implicated in cancer invasion (18), and urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) are validated prognostic and predictive markers for breast cancer (19).

The regulation of matrix proteolysis is complex and can involve the concerted action of multiple proteinases and proteinase classes from both tumor cells and adjacent resident and infiltrating cells (Figure 19-5). The conversion of pro-MMP2 to active MMP2 requires membrane-type MT1-MMP (MMP14), a transmembrane MMP that is activated intracellularly by the proprotein

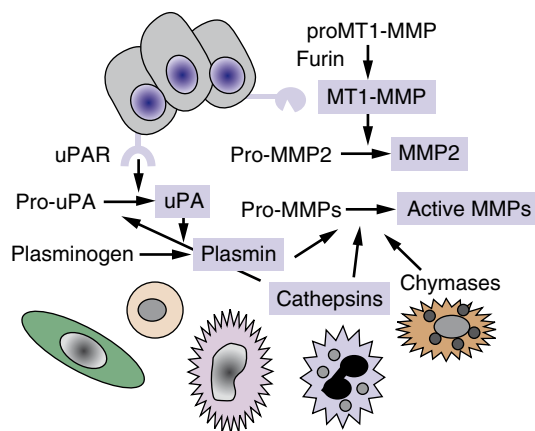


FIGURE 19-5 PROTEOLYTIC CASCADES. Extracellular proteinases are made by tumor cells as well as by stromal fibroblasts and inflammatory cells. Proteolytic cascades result in the conversion of pro-enzymes to their active form. Enzymes in blue boxes are capable of degrading components of the extracellular matrix (ECM). In many cases, proteolytic cascades are localized to the surface of tumor cells. The urinary plasminogen activator receptor (uPAR) is expressed by many tumor cells and initiates and localizes the conversion of pro-urokinase plasminogen activator (pro-uPA) to its active form, which then converts the serum protein plasminogen into the active serine proteinase, plasmin. The membrane type 1-matrix metalloproteinase (MT1-MMP) is a transmembrane protein that is activated intracellularly by the proprotein convertase furin. MT1-MMP converts pro-MMP2 to its active form, MMP-2. Enzymes of many classes convert pro-MMPs to their active form.

convertase family member, furin. There is evidence for a cascade of cathepsin-D–cathepsin-B–uPA–plasmin–MMP activation that results in activated enzymes capable of degrading all components of the ECM. Proteolysis is also regulated by the production of specific endogenous protease inhibitors, including the tissue inhibitors of metalloproteinases (TIMPs), serine proteinase inhibitors (serpins), and cysteine protease inhibitors (cystatins). These inhibitory activities are produced and secreted by tumor or stromal cell types, and some proteinase inhibitors are stored in high concentrations in the ECM. Proteinase activity cascades can function via proteolytic degradation of some of these proteinase inhibitors in addition to activation of other proteinases.

The original view that proteolytic enzymes function predominantly to remove physical ECM barriers has been expanded with the realization that proteolysis is a key regulator of multiple steps of tumor progression. For example, MMP substrates in the matrix or on the cell surface that modulate cellular growth, differentiation, apoptosis, angiogenesis, chemotaxis, and migration have been identified (17). The abundant evidence for a role for MMPs in tumor progression led to the design and testing of synthetic MMP inhibitors for cancer therapy (20). These inhibitors proved to be ineffective in clinical trials, results that have been explained by problems with inhibitor or clinical trial design and a lack of understanding of the broad range of MMP activities resulting in both cancer-promoting and cancer-inhibitory effects.

Motility

Cellular locomotion occurs as the result of coordinated polymerization and depolymerization of the actin cytoskeleton to extend a pseudopod at the leading edge of the cell, followed by contraction associated with disassembly of cell–matrix adhesive contacts at the trailing edge (21). Lamellipodial protrusions at the leading edge are nucleated by a branched actin network involving the Arp2/3 complex and its regulators, the WASp (Wiskott-Aldrich syndrome protein) family, cortactin, and the GTPase Rac. Actin contractility is regulated by myosin light-chain kinase and upstream small GTPases, in particular Rho and its effector Rho-kinase (ROCK). Single cells migrate with a spindle-shaped morphology, referred to as mesenchymal migration, or with the less-adhesive ellipsoid shape used by leukocytes and *Dictyostelium* termed “amoeboid migration” (Figure 19-6). Collective migration can occur when the cells retain cell–cell junctions and clusters of cells move in single file through a tissue.

Tumor cells can secrete factors that stimulate motility in an autocrine fashion. Tumor cell–produced lysophospholipase D (autotaxin) stimulates motility, as does lysophosphatidic acid (LPA), which can be produced by lysophospholipase D activity on lysophosphatidylcholine. Hepatocyte growth factor/scatter factor (HGF/SF) interacts with its receptor, *c-met*, to induce chemokinetic activity of epithelial cells, resulting in an invasive phenotype. Directional motility is a chemotactic or haptotactic effect in response to a gradient of soluble or localized factors, respectively. Chemotaxis is often the result of growth factors such as IGF, and chemokines of the CCR and CXC family. Haptotaxis

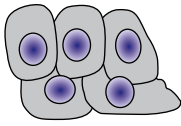
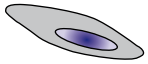
	Collective	Mesenchymal	Amoeboid
			
Cell:cell contact	+	–	–
Cell:matrix contact	+	+	–
Proteolysis	+	+	–
Motility	+	+	+

FIGURE 19-6 TYPES OF CELLULAR INVASION. Cells can move through matrix barriers as collectives, in which multiple cells remain attached and move together, or as single cells with mesenchymal or amoeboid characteristics. Epithelial-derived tumor cells undergoing collective migration retain cell–cell adhesions, whereas those undergoing mesenchymal or amoeboid movement have reduced or absent cadherin-mediated adhesions. Mesenchymal motility requires proteolysis and integrin-mediated cell–matrix adhesion. In the absence of proteolysis and extracellular matrix (ECM) adhesions, tumor cells can move through ECM using amoeboid movement, similar to that displayed by infiltrating leukocytes. Amoeboid movement is characterized by elevated actin cytoskeleton activity mediated by the small GTPase Rho and its regulator Rho-kinase.

is characterized as a response to gradients of ECM components such as laminin-5 and fibronectin and can be modulated positively or negatively by proteolysis.

Coordination of Cancer Invasion

The coordination of cell–cell and cell–matrix adhesion, matrix degradation, and cytoskeletal activity is required for cellular invasion. The type of cell migration (i.e., collective, mesenchymal, or amoeboid) is influenced by the relative levels of adhesion mediated by cadherins and integrins, proteolytic activity, and actin contractility. Modulation of any of these factors can convert one type of motility into another (21).

Invadopodia is the name that has been given to structures identified in invading cells that represent the physical convergence of the adhesive, proteolytic, and motility component of invasion (Figure 19-7; 22). Invadopodia are actin-rich organelles that protrude from the plasma membrane and contact and locally degrade the ECM. Invadopodia contain adhesion molecules, including several $\beta 1$ integrins and CD44, the serine proteinases seprase and dipeptidyl dipeptidase IV, and several MMP and ADAM metalloproteinases. Inside the plasma membrane, invadopodia contain actin and actin assembly molecules and multiple signaling molecules including focal adhesion kinase (FAK), *src* associated proteins such as p130Cas and Tks5/FISH (tyrosine kinase substrate 5/five SH3 domains), and the small GTPases cdc42, Arf1, and Arf6. Thus, invadopodia are implicated as key cellular structures that are used to coordinate and regulate the various components of the process of cancer invasion.

The Metastatic Cascade

Although invasion is required for metastasis, the ability to invade is not sufficient for metastasis (Figure 19-1). Some tumors are highly aggressive, forming secondary lesions with high frequency (e.g., small cell carcinoma of the lung, melanoma, pancreatic

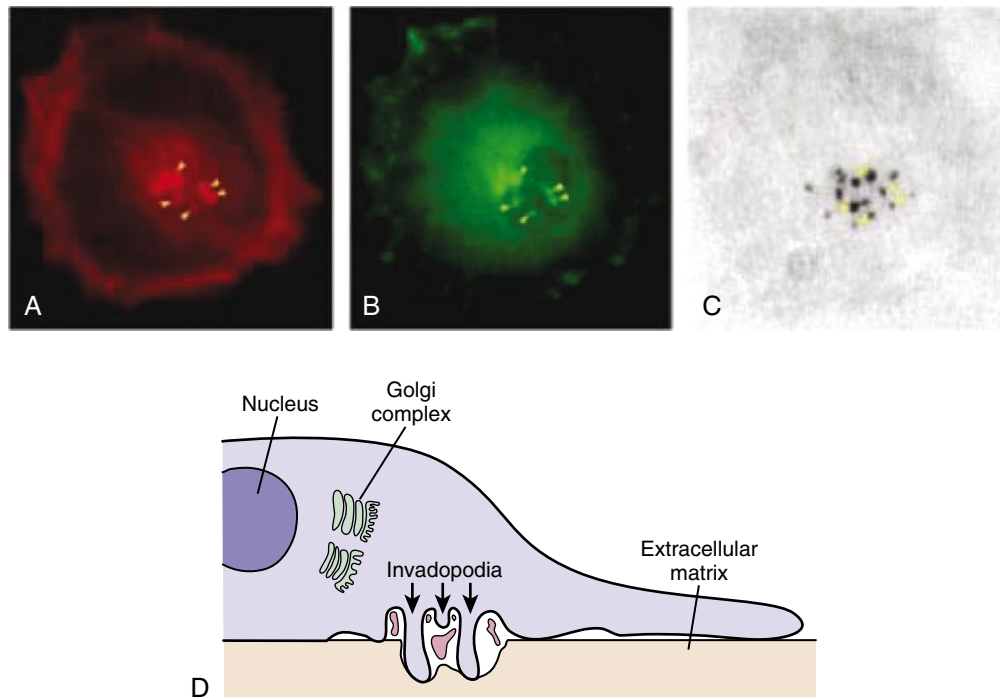


FIGURE 19-7 INVADOPIEDIA. Confocal laser image showing triple immunofluorescence labeling of A375MM melanoma cells plated on tetramethylrhodamine isothiocyanate (TRITC)-conjugated gelatin. **A:** Invadopodial structures marked by actin-binding phalloidin-Alexa 546. **B:** Invadopodial structures marked by Alexa 633-conjugated anti-phosphotyrosine antibodies. **C:** Degradation areas on the underlying Alexa 488-conjugated gelatin. Arrowheads indicate the colocalization between actin, phosphotyrosine, and patches of degraded extracellular matrix, fulfilling the criteria for the definition of invadopodia. **D:** Schematic diagram of the invadopodial complex based on correlative light-electron microscopy reconstructions. Spatial relationships with the nucleus and the Golgi complex are shown. Invadopodial protrusions originate from profound invaginations of the ventral surface of the plasma membrane; within the area delimited by the large invagination, large fragments of gelatin can often be seen. (From Ayala I, Baldassarre M, Caldieri G, et al. *Invadopodia: a guided tour*. *Eur J Cell Biol* 2006;85:159, with permission.)

carcinoma), whereas others are rarely metastatic despite being locally invasive (e.g., basal cell carcinomas of the skin, glioblastoma multiforme). Fidler and colleagues have proposed an analogy regarding metastasis that is highly illustrative. Metastatic cells are likened to athletes participating in the decathlon. Each cell must be capable of completing every step of the metastatic cascade. If a cell cannot complete any step, it cannot go on to subsequent steps and cannot form a metastasis.

Metastasis is primarily thought of developing via dissemination in the bloodstream, although other routes of spread occur. Carcinoma cells tend to escape and spread initially to draining lymph nodes, becoming trapped and proliferating. The thoracic duct links the lymphatic system to the blood stream, connecting lymphatic to hematogenous spread. Metastases can also develop by spreading across body cavities. For example, ovarian carcinoma cells most frequently establish secondary tumors by dissemination in the peritoneum while rarely forming metastases via hematogenous spread. Other routes of spread also exist but are far less common (e.g., dissemination of melanoma cells along the space between endothelium and basement membrane or perineural spread in pancreatic and prostatic carcinomas). Thus, the route of dissemination is not inherent to a definition of metastasis.

Intravasation

How tumor cells enter the blood stream is not clearly understood. The growth of a tumor exerts a hydrostatic pressure, and studies

imply that tumor cell invasive cords follow lines of least resistance. Angiogenesis is likely to be a prerequisite for metastasis, but this has not been formally proven (see Chapter 18). Tumor cell entry into intact blood vessels is an active process that requires serine- and metalloproteinase activity in an experimental model of intravasation (23). Tumor blood vessels, however, are highly abnormal with fewer pericytes and increased permeability compared with normal vessels, and presumably provide an easier route for direct entry into the blood stream (24). Lymphatic vessels are also abnormal, but their role in intravasation is unknown. Regardless of the route, tumor cells enter the circulation in great numbers: Estimates are 3 to 4 million cells/day/g of tumor (25). The number of tumor cells in the peripheral blood, however, does not predict if the patient will develop metastases (26). In contrast, the detection of disseminated tumor cells in lymph nodes and bone marrow does correlate with metastatic relapse, suggesting that, at least in breast cancer, the properties that allow the cells to find their way to these tissues and survive are the same properties that permit distant metastases.

Transport

Once tumor cells enter a circulatory compartment, they can move actively by motility mechanisms or passively, carried or pushed along with fluid flow. Injection of radiolabeled cells directly into circulation reveals that a substantial proportion is lost during the transport phase of the metastatic cascade. Many tumor cells are eliminated by

natural killer (NK) cells or monocytes before arrival in a secondary site. Tumor cells that escape immune recognition are frequently killed by exposure to hemostatic shear forces (27). Bioassays in the lungs, liver, heart, and muscle have been performed following intravenous injection of tumor cells. It is noted that by the time it takes to remove the tissues for assay (2–3 minutes), most cells are dead due to mechanical trauma (27). The average tumor cell diameter ranges from 20 to 30 μm but must navigate through vessels significantly smaller (e.g., 6- to 7- μm capillaries). Even if tumor cells have the ability to deform and squeeze through the passages they are subjected to significant hydrostatic pressures. Depending on the tumor type and biophysical parameters such as membrane fluidity, cellular elasticity, and cytoskeletal organization, the cells will remain intact or be broken by shear. Deformability is also impacted by the pressures found within various tissues. In contrast to the shear forces usually encountered in the vasculature, blood flow in bone sinusoids is sluggish (≈ 30 -fold lower than capillaries and postcapillary venules), and diameter is not a concern (28).

During transport, the behavior of tumor cells is often determined by their presence as single cells or as emboli. Embolization can be homotypic (tumor cell–tumor cell) or heterotypic (tumor cell–leukocyte, tumor cell–platelet, tumor cell–fibrin). The association of tumor cells with blood cells can be the result of altered cell surface glycosylation and expression of sialyl Lewis X/A on the tumor cell that permits interaction with a class of vascular adhesion molecules found on normal leukocytes and endothelium, the selectins. Alterations in the adherence of tumor cells to endothelium via E-selectin, platelets via P-selectin, and leukocytes via L-selectin alter metastatic potential in animal models (29). Embolus size can also contribute to protection of the tumor cells from biophysical forces or immune attack. In essence, encapsulation of tumor cells helps to protect them. As a result of the consequence of emboli formation, heparin, an inhibitor of selectin/glycan interactions, has been considered as an antimetastatic agent.

Visualization of tumor cells in the circulation during transport indicates that the cells roll rather than float in a manner analogous to leukocytes. Nonetheless, during this time, tumor cells are weakly adherent and subject to anoikis, a specialized type of apoptosis in which cells that are anchorage-dependent are induced to die (30). In general, metastatic cells are more resistant to anoikis than nonmetastatic cells and are frequently referred to as being anchorage-independent. This is somewhat misleading, because some tumor cells will induce apoptosis even if firmly attached to a substrate if that substrate is not the preferred one for the type of cell. It is possible, then, that circulating tumor cells receive sufficient signals from the extracellular matrix, other cells, and/or serum proteins to limit their susceptibility to anoikis.

Arrest

It is important to discriminate between the physical trapping and arrest of circulating cells in the microvasculature and selective adhesion to the walls of the microvasculature. Both processes have been observed, and the relative importance of these mechanisms in specific organs is debated.

There are three types of endothelial structures found in higher vertebrates: continuous, discontinuous, and fenestrated. Most endothelial cells form tight junctions with their neighbors and have a continuous, unbroken basement membrane beneath them. However in certain organs, such as liver and spleen, the endothelial cells and the basement membrane have gaps, or discontinuities, in their structure. In the kidney, a fenestrated endothelium, there are gaps between endothelial cells but a membrane-like structure connects them and the entire structure overlaps in a continuous basement membrane. The structure of these endothelial/basement membrane barriers contributes to the normal function of the tissues and forms different barriers through which tumor cells must pass.

Adhesion of circulating tumor cells to organ microvessel endothelial cells represents one of the more important steps in metastasis, especially organ-specific metastasis. In general, higher rates of tumor cell–endothelial adhesion correlate well with metastatic potential. *In vivo* and *in vitro* kinetic studies indicate that initial attachment of cancer cells occurs preferentially at endothelial cell junctions (31). Frequently, tumor cells adhere at sites where inflammation is taking place, and is most likely related to alterations in cell surface components of endothelial cells at these sites. Tumor cells use many of the same mechanisms to attach to and traverse endothelium as inflammatory cells, including glycan/selectin interactions.

Once tumor cells bind to the endothelium, they induce the endothelial cells to retract and eventually overlap the tumor cell. During this time, there is no loss of electrical resistance, suggesting that tight junction integrity is maintained. Tumor cells then adhere to subendothelial basement membrane components, and a higher rate of tumor cell adhesion to subendothelial basement membrane correlates with metastatic potential. In the case of HT1080 fibrosarcoma cells, the attachment of circulating tumor cells to the lung vasculature is mediated by tumor $\alpha_3\beta_1$ integrin ligation to laminin-5 in the basement membrane (32). Patches of exposed basement membrane were found to be preexisting using intravital microscopy techniques in isolated, perfused lungs.

Arrested tumor cells can undergo rapid apoptosis. It is envisioned that in some cases this is the result of the lack of suitable survival signals and the initiation of anoikis. In addition, the attachment of tumor cells to endothelium can release nitric oxide (NO) produced by endothelial nitric oxide synthase (33). NO can induce apoptosis of tumor cells, indicating an active process that contributes to tumor cell loss and metastatic inefficiency.

Extravasation

Extravasation is the process of tumor cells invading from the interior of a vessel into the organ parenchyma. Extravasation was viewed as a rate-limiting step for metastasis formation, but intravital microscopy studies have indicated that extravasation can be a remarkably efficient process, at least in some situations. For example, 87% of B16F1 murine melanoma cells that were injected through the mesenteric vein into the liver were arrested in the liver 90 minutes after injection and 83% of the injected cells were found in the liver parenchyma by 3 days, indicating that more than 95%

of the arrested cells extravasated (34). The molecular mechanisms underlying extravasation are viewed as being identical to those involved in invasion, and in vitro assays for extravasation reveal a contribution of cellular adhesion molecules, proteinases, and motility factors.

There is controversy as to whether extravasation is required for the formation of metastases. In the case of some pulmonary metastases, there is evidence that tumor cells can attach to the lung endothelium, survive, and grow intravascularly (35). Extravasation occurs in this model only when the intravascular foci outgrow the vessel.

Colonization

Colonization, the formation of clusters of tumor cells at ectopic sites, represents a highly inefficient step in the metastatic cascade. In the model of B16F1 cells injected into the liver vasculature, only 2% of the injected cells formed micrometastases, and only 0.02% formed lesions that persisted, grew progressively, and threatened the life of the animal (34). The formation of micrometastatic lesions requires that the tumor cell must first survive and then grow in the foreign environment. In some tumor types (i.e., breast and melanoma), metastases can arise decades after the treatment of the primary tumor, indicating that tumor cells can survive in a state of dormancy for long periods. Tumor cells can persist as solitary cells, or they can grow to a size of several hundred cells in which the rate of growth is balanced by the rate of apoptosis. Conversion to a clinically detectable metastatic lesion requires the subsequent initiation of angiogenesis (see Chapter 18). The growth of the cells is dependent on factors, primarily soluble growth factors, present at the site of colonization. Although it is natural to focus on factors that promote the growth of tumor cells in selective sites, there is ample experimental evidence showing that some tissues are hostile to tumor cells.

A tumor cell’s ability to establish a metastatic lesion is very much dependent on the microenvironment (see Chapter 17). A prime example of this effect is the role of the “vicious cycle” in the propensity for breast carcinoma to metastasize to bone (Figure 19-8; 36). The mammary carcinoma cells produce parathyroid hormone–related peptide (PTHrP), which during pregnancy would function to release calcium from bone stores. Using the same molecular pathways, tumor cell–produced PTHrP acts on its receptors on osteoblasts to release the tumor necrosis factor- α (TNF- α) family member, receptor activator of nuclear factor- κ B ligand (RANKL). RANKL interacts with its receptor RANK on osteoclasts and activates them to degrade mineralized bone. The bone matrix contains an abundance of growth factors, including PDGF, FGFs, IGF-1, and TGF- β /bone morphogenetic protein family members, which are released during the osteolytic process. It is the release of these growth factors that stimulates the breast cancer cells to growth and to continue to secrete PTHrP and fuel the “vicious cycle.” The colonization of breast cancer cells in the bone is thus facilitated by specific characteristics of the bone microenvironment that promote the growth of breast cancer cells.

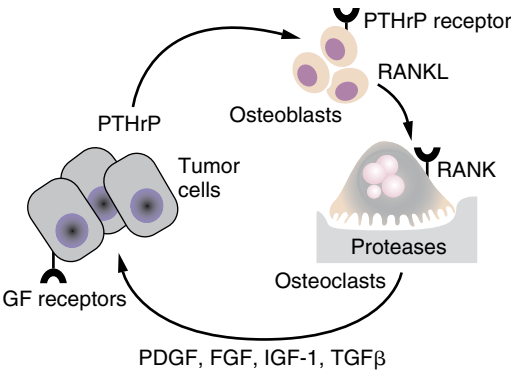


FIGURE 19-8 THE VICIOUS CYCLE OF HOST–TUMOR INTERACTIONS IN BREAST CANCER METASTASIS TO BONE. Breast cancer cells produce parathyroid hormone related protein (PTHrP), which stimulates bone osteoblasts to express the tumor necrosis factor- α (TNF- α) family member receptor activator of nuclear factor- κ B ligand (RANKL). RANKL interacts with its receptor RANK on osteoclast precursors to differentiate into active osteoclasts, resulting in the release of proteases and bone degradation. Growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF-1), and transforming growth factor- β (TGF- β), which are stored in the bone matrix, are released and stimulate the growth of receptor-containing tumor cells. An increase in tumor cells results in an increase in PTHrP release, leading to a vicious cycle of tumor cell growth and bone degradation.

Organ Selectivity of Metastasis

There is a clear tendency for primary tumors to form metastatic lesions in specific organ sites (Table 19-1). Common regional metastatic involvements can often be attributed to anatomic or mechanical considerations (e.g., efferent venous circulation or lymphatic drainage) and explained by arrest of tumor cells in

Table 19-1 Common Sites of Metastasis

Primary Tumor Site	Most Common Sites of Metastases
Breast	Axillary RLN, contralateral breast via lymphatics, lung, pleura, liver, bone, brain, adrenal, spleen, ovary
Colon	RLN, liver, lung, direct extension into urinary bladder or stomach
Kidney	lung, liver, bone
Lung	RLN, pleura, diaphragm by direct extension, liver, bone, brain, kidney, adrenal, thyroid, spleen
Ovary	Peritoneum, RLN, lung, liver
Pancreas	Liver, stomach by direct extension, colon, peritoneum
Prostate	Bones of spine and pelvis, RLN
Stomach	RLN, liver, lung, bone
Testis	RLN, lung liver
Urinary bladder	Direct extension into rectum, colon, prostate, ureter, vagina, bone, RLN, bone, lung, peritoneum, pleura, liver, brain
Uterine endometrium	RLN, lung, liver, ovary

RLN, regional lymph nodes.

the first capillary bed or lymph node encountered (37). Since most tumor cells enter the vasculature in small veins or capillaries, the most common site of metastasis is lung and liver. However, distant metastasis patterns are typically more site specific. In 1889, Paget analyzed postmortem data of women who died of breast cancer and noticed a higher frequency of metastasis to skeleton than would be expected based solely on cardiac output to each organ (38). He concluded that the pattern of organ distribution of metastases was not simply a matter of chance and suggested that metastases develop only when the “seed” (tumor cells with metastatic ability) and the “soil” (organs or tissues providing growth advantages to seeds) are compatible. Importantly, the mechanical theory and the seed and soil hypothesis are not mutually exclusive, and both contribute to metastatic dissemination.

Experimental data supporting the “seed and soil” hypothesis include preferential invasion and growth of B16 melanoma metastases in specific organs (39). In addition, palliative treatment of women with advanced ovarian carcinoma has provided an opportunity to test this theory in humans. These patients often have a large ascites burden, but seldom present with disease outside the peritoneal cavity. Tarin and colleagues treated patients with potentially lethal malignant ascites by introducing a tube that drains the peritoneal ascites into the vena cava (40). In doing so, tumor cells in the ascites were given direct entry into the circulation. Despite continuous entry of billions of viable tumor cells into the circulation, metastases to the lung (i.e., the first capillary bed encountered) were rare. This single clinical observation highlights the inefficiency of the metastatic process and, more important, demonstrates that merely seeding cells in different tissues is not adequate to develop metastases.

The mechanisms responsible for organ selectivity in tissues can be attributed to the arrest and the colonization steps of the metastatic cascade in particular. Tumor cells adhere more selectively to organ-derived microvascular endothelial cells than large-vessel endothelial cells, and variants of the B16 melanoma previously selected for metastases to brain, lung, ovary, or liver adhere at a more rapid rate to brain, lung, ovary, or liver endothelial cells, respectively (31). Using phage-display technology, endothelial cells in different tissues have been demonstrated to express unique markers, and tumor cells recognize the molecular “addresses” to adhere in a selective manner (41). Tumor cells are also able to recognize subendothelial basement membrane differences. In vitro studies demonstrate the selective growth of tumor cells in organ-derived soluble growth factors or cells (42). In vivo, breast tumor cells that express the chemokine receptor CXCR4 preferentially metastasized to tissues that expressed the ligand SDF1/CXCL12 (43). There is a concept that tumor cells colonize in a premetastatic niche initiated in target organs by tumor cell-generated soluble factors that induce the expression of fibronectin by resident fibroblast-like cells (44). Bone marrow-derived cells that express the vascular endothelial cell growth factor receptor 1 and the integrin $\alpha_4\beta_1$ selectively adhere to these regions, produce the proteinase MMP9 and the chemokine SDF1/CXCL12, and provide a permissive niche for the colonization by tumor cells.

Although the data strongly support the notion that there are soluble factors produced in different tissues to which tumor cells can respond, the process of homing has not been validated. Strictly speaking, homing would require directed movement throughout the transit of tumor cells as they leave the primary tumor. Rather, tumor cells distribute according to circulatory patterns initially but may “home” once they are more proximate. Many of the mechanisms used by lymphocytes to home to peripheral lymph nodes or sites of inflammation are apparently shared by tumor cells.

Some of the strongest evidence supporting organ selectivity of cancer cells comes from data showing selection of variants that colonize different tissues. The first selections were done by repetitive isolation of lung metastases from the B16 melanoma followed by reinjection and recolonization (39). Similar approaches have been used for other tumors, most recently using a human breast carcinoma cell line with selection of metastases to bone, lung, and adrenal gland. Using these breast carcinoma cell lines coupled with comparison by cDNA microarray has highlighted the requirement for coordinated expression of multiple genes for metastasis (45). Transcriptomes were compared between parental and bone-selective variants and over and underexpressed genes were identified. Among the overexpressed genes in the bone metastasis signature were a matrix metalloproteinase, MMP1; the ECM component osteopontin; the cytokine interleukin-11; the chemokine receptor, CXCR4; and connective tissue-derived growth factor. Subpopulations within the parental population expressed one or more of the bone signature genes but only a few expressed all of them. Transfection of individual cDNAs only modestly increased bone metastatic efficiency, whereas cotransfection of gene combinations into the parental cells resulted in populations as efficient at bone colonization as the bone-selective variants. Similar studies with a lung-selective variant revealed a lung metastasis signature that overlapped only minimally with the bone metastasis signature (46). These data highlight that there are specific genes that control metastasis in an organ-specific fashion, and coordinated expression of multiple genes is required.

Genetic Determinants of Metastasis

Primary tumor formation and metastasis are distinct processes. Locally growing tumors can grow and progress without the development of metastases. This observation prompted the hypothesis that the molecular processes regulating tumorigenicity and metastasis are distinguishable. The existence of metastasis-controlling genes is supported by data from several laboratories showing that specific cDNAs block metastasis but not tumorigenicity (47,48). Such genes, by definition, are called metastasis suppressors. Metastasis suppressors are distinct from tumor suppressors. Tumor suppressors block both tumor formation and metastasis since the former is prerequisite to the latter.

The identification of *nm23*, the first metastasis suppressor, provided functional evidence for the existence of molecules that specifically regulate metastasis (49). Subsequently several laboratories have used various unbiased approaches to identify

metastasis suppressors. The use of *in vivo* assays is required because *in vitro* assays are often of inadequate complexity to sufficiently model the entire process of metastasis. Further, there are currently no *in vitro* models that allow study of preferential growth within different target tissues. Table 19-2 lists the proteins that have bona fide metastasis suppressor activity *in vivo* (i.e., suppression of metastasis following ectopic expression into metastatic cell lines). Metastasis suppressors vary widely in their cellular locations and biochemical functions and many would not have been predicted *a priori* on the basis of their known cellular function(s)(47).

Many metastasis suppressors are involved in cellular responses to exogenous signals, highlighting the importance of tumor–stromal interactions. Cells respond to external stimuli in a spatiotemporal manner by using a relatively limited number of signaling pathways. In this light, metastasis formation can be viewed as the result of a tumor cell’s ability to respond to multiple growth milieus as opposed to being restricted to growth at orthotopic sites.

In addition to the genetic changes associated with the metastatic cell, the importance of the host genome has been demonstrated by Hunter and colleagues (50). Transgenic mice expressing the polyoma middle T oncogene under control of the mouse mammary tumor virus promoter develop metastatic mammary tumors. When bred to nonsyngeneic mice, metastatic potential was enhanced or inhibited, depending on the strain of mouse. Because all tumors were initiated by the same oncogenic event, differences in metastasis are explained by genetic background differences and specific loci contributing to metastatic efficiency have been identified. Using the decathlon analogy, the difficulty of the course is determined by host genetics, whereas the ability of the athlete to overcome the obstacles is assisted by the plasticity of the tumor genome.

The metastatic potential of a primary tumor can be determined by gene expression profiling. A 70-gene signature that distinguished lymph-node–negative breast cancer patients with a high or low probability of remaining free of distant metastases over a 12-year period was identified (51). Clinical trials with this approach, as well as trials using a commercially available polymerase chain reaction (PCR)–based 21-gene profile assay, have indicated an advantage in identifying women who are likely to derive clinically significant benefit from chemotherapy to prevent the occurrence of breast cancer metastasis (52).

Table 19-2 Metastasis Suppressors

Nm23-H1	KISS1	JNKK1/MKK4
BRMS1	MKK6	Cadherin-11
RKIP	Gelsolin	E-cadherin
Drg1	KAI1	N-cadherin
CD44	RECK	Caspase-8
CTGF	RhoGD12	Claudin-1
	SSECKS	Claudin-4

The Tumor Microenvironment in Metastasis

Metastasis is regulated by tumor and stromal interactions at every step (see Chapter 17). Tumor cells can co-opt endothelial cells to create an unimpeded vascular supply. Many of the proteases responsible for tumor cell invasion appear to be produced by stromal cells rather than the tumor cells. Tumor-associated fibroblasts can stimulate tumor cell growth and/or invasion whereas normal fibroblasts (i.e., fibroblasts isolated at a distance from the tumor) are growth neutral or growth suppressing. Adhesion to endothelium and growth in response to organ-specific factors are dictated by microenvironmental cues. In experimental models, the ability of a primary tumor to metastasize is dependent on the site of injection. For example, human colon cancer cells injected subcutaneously do not metastasize, although the same cells colonize the liver following orthotopic injection into the gastrointestinal tract (53). Although mechanical factors contribute to this effect, molecular differences in the tumor microenvironment are contributing factors.

Infiltrating immune and inflammatory cells have dual effects on tumor metastasis. On one hand, recognition by the immune system that a tumor cell is “foreign” often leads to the destruction of the tumor cell in the elimination of the cancer (54). However, tumor cells subvert the immune system and often take advantage of properties inherent to the inflammatory cells, such as invasion. Tumors induce macrophage and neutrophil infiltrates that have been associated with increased invasion across ECM *in vitro*. Coinjection of tumor elicited macrophages or neutrophils increased metastasis in animal models as well (55,56). The inflammatory cells invaded through basement membranes followed by tumor cells. In addition, the inflammatory system in various tissues produces factors that are stimulatory or inhibitory for tumor growth.

Therapeutic Challenges and Opportunities

How can our understanding of the molecular basis of metastasis lead to improved cancer therapies? First, one can ask what is unique about metastatic cells that distinguish them from normal cells. Unfortunately, the answer is not much. Cellular behavior in metastatic cells is governed by the same mechanisms that are present in normal cells and under normal physiology. The ability to invade is not unique to cancer cells. Leukocytes and neurons invade tissues as part of inflammation and normal development, respectively. Similarly, leukocytes and stem cells exhibit intermittent adhesion as part of their normal function. And while moving around the body, they are certainly resistant to anoikis. These cells exert influence on the secondary site. During inflammation, for example, leukocytes and fibroblasts degrade and reconstitute extracellular matrix. Proliferation of cells at two different locations would seemingly distinguish metastatic cells from normal counterparts; however, macrophages and stem cells (e.g., angioblasts) can proliferate at secondary sites, and in the case of stem cells, this is a

persistent proliferation. Together, the distinctions between metastatic tumor cells and normal cells are difficult to identify. Making matters even more complicated, metastatic cells use essentially the same molecular mechanisms for accomplishing each of the steps as do their normal counterparts.

There are some essential differences between normal cells and metastatic cells. Importantly, all of the properties necessary for metastasis must coexist within a single cell since metastases arise predominantly from single cells (27,28). Conceptually, this offers opportunities for synergy in therapeutic approaches while minimizing side effects. Molecules that block adhesion to organ-specific endothelium and the underlying basement membrane offer some specificity in response. Invadopodia appear to be specialized structures found in only a limited number of normal cells and present an opportunity for selective targeting. In addition,

unlike stem cells that can enter a secondary site, proliferate, and differentiate, metastatic cells do not differentiate fully at a secondary site. Hence, another hallmark of metastatic cells is their ability to persistently proliferate without fully differentiating.

The therapeutic opportunity for controlling metastasis may not rest in understanding the unique characteristics of the tumor cell as much as in understanding the control exerted by the tumor microenvironment. Targeting normal cells, as opposed to genetically unstable tumor cells, lessens the chance of drug resistance. The colonization stage of metastasis offers exceptional therapeutic opportunities, since the cells can remain alive but dormant or pre-angiogenic for long periods of time. Understanding how the tumor microenvironment maintains dormancy, or promotes the conversion to a clinically detectable metastasis, could provide intriguing therapeutic possibilities.

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Cancer Genomics

A central tenet in cancer etiology is that a cancer evolves from a single, normal cell as it accumulates an ensemble of genomic and epigenomic aberrations that collaborate to develop the eventual malignant phenotype. Substantial effort in the past several decades has focused on identifying these aberrations. Classically, these genes have been classified as tumor suppressors whose inactivations promote cancer and oncogenes whose activations or overexpressions promote cancer. More recent genome-wide analyses of the epigenome and genome have shown that this process is far more subtle and complicated; revealing a wide range of molecular abnormalities that contribute to cancer pathophysiologies such as loss of ability to properly differentiate, increased genome instability, reduced apoptosis, reactivation of telomerase, insensitivity to antigrowth factors, ability to invade and metastasize, and sustained angiogenesis (1). Since these molecular defects influence tumor behavior and hence clinical outcome, assays for abnormalities are often used as indicators of survival duration and therapy response and/or as markers for early cancer detection. In addition, the abnormalities may be targets for therapeutic interventions that attempt to reverse or correct the abnormalities.

This chapter focuses on DNA-level genomic and epigenomic abnormalities with particular emphasis on analysis technologies that enable experimental and clinical studies. We include genomic and epigenomic abnormalities because these typically collaborate to enable cancer genesis and progression (2). In some cases, the structural epigenomic and genomic events may contribute at different stages of cancer development. Genomic and epigenomic events exert their influences transcriptionally and translationally with the possibility of feedback between them as illustrated in Figure 20-1.

Aberration Types

The Genome

Genomic abnormalities contribute to cancer pathophysiology by altering transcription levels of genes or regulatory noncoding RNAs (ncRNAs) and/or by changing gene function. Examples of typical abnormalities summarized in Figure 20-2 include changes in genome copy number that result from gains and losses of single copies of genome regions, high-level amplification, homozygous deletions, mutations that alter coding sequences, regulatory regions

or message stability, and chromosome rearrangements that alter gene or chromatin structure.

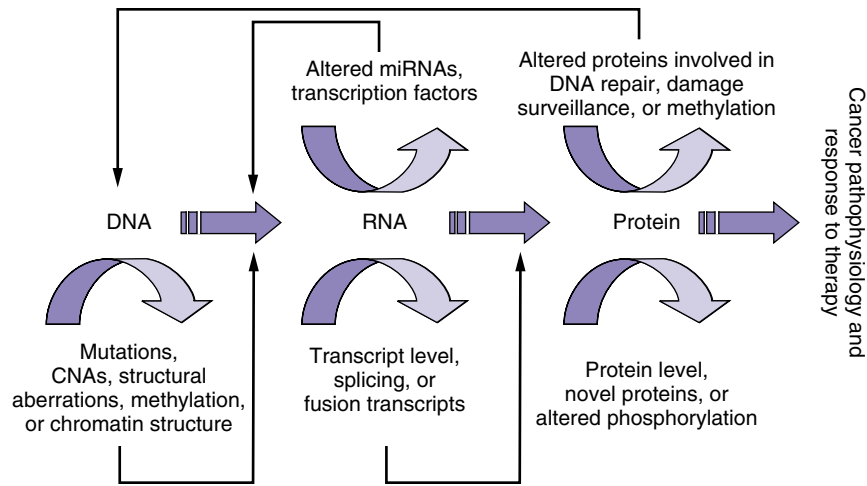
Structural Aberrations

Structural aberrations—especially translocations—are the most well-established genomic abnormalities and have long been associated with outcome and treatment response in leukemias and lymphomas. Prominent examples discovered using classical karyotyping procedures include the t(9;22) translocation that fuses the *BCR* and *ABL* genes in chronic myelogenous leukemia and acute lymphoblastic leukemia (3), the t(14;18) translocation that juxtaposes *BCL2* and the immunoglobulin heavy-chain gene (*IgH*) in follicular lymphoma (4), and the t(8;21) and t(15;17) translocations that fuse *AML1* to *ETO* (5) and the *RARA* to *PML* (6), respectively, in acute myeloid leukemia. Structural aberrations also have been extensively catalogued for solid tumors but until recently, individual structural aberrations have not been shown to be sufficiently prevalent to catch much attention. The recent exception is the discovery of the *TMPRSS2* and *ETS* transcription factor genes in prostate cancer. This fusion event occurs in over 70% of prostate cancers (7).

Copy Number Aberrations

Several high-level gene amplification events have been associated with the pathophysiology of human tumors. These include amplification of *NMYC* in neuroblastoma (8); *ERBB2*, *EMSY*, and *CCND1* in breast cancer (9–12); *AR* in prostate cancer (13); *AKT* (14); *RAB25* in ovarian cancer (15); *EGFR* in glioblastoma and lung cancers (16,17); *PIK3CA* in ovarian and lung cancer (18,19); and *MYC* in a broad range of tumors (20). Deletions also contribute significantly to tumor development by contributing to gene inactivation. Examples include *TP53* in many tumors (21), *RB1* in retinoblastoma (22), *BRCA1* in breast and ovarian cancer (23), and *PTEN* (24) and *CDKN2A* (25) in a broad range of tumors. However, many other regions of amplification and lower level copy number abnormality in human cancers—especially solid tumors—have been revealed using comparative genomic hybridization (CGH). CGH allows copy number abnormalities to be mapped onto a normal representation of the genome for simple interpretation. This technology has made it clear that there are many regions of recurrent high-level amplification and homozygous deletion and, equally important, that lower level recurrent copy

FIGURE 20-1 Schematic illustration of how aberrations involving the genome, transcriptome, and proteome collaborate during the development of cancer pathophysiology.



number increases and decreases involving thousands of genes are common in most solid tumor types. Some studies have suggested that low-level deregulation of many of these genes contributes to an increase in general metabolism (26) whereas recurrent high-level amplifications, homozygous deletions, translocations, and mutations alter genes that contribute more directly to one or more aspects of cancer pathophysiology known as cancer hallmarks (1). Interestingly, genes activated by translocations in leukemias and

lymphomas often are activated by amplifications or mutations in solid tumors; perhaps because mechanisms of genome instability or DNA repair differ between these tumor types.

Mutations

Somatic mutations involving several genes have been found to be important in selected human cancers. In general, the frequency and

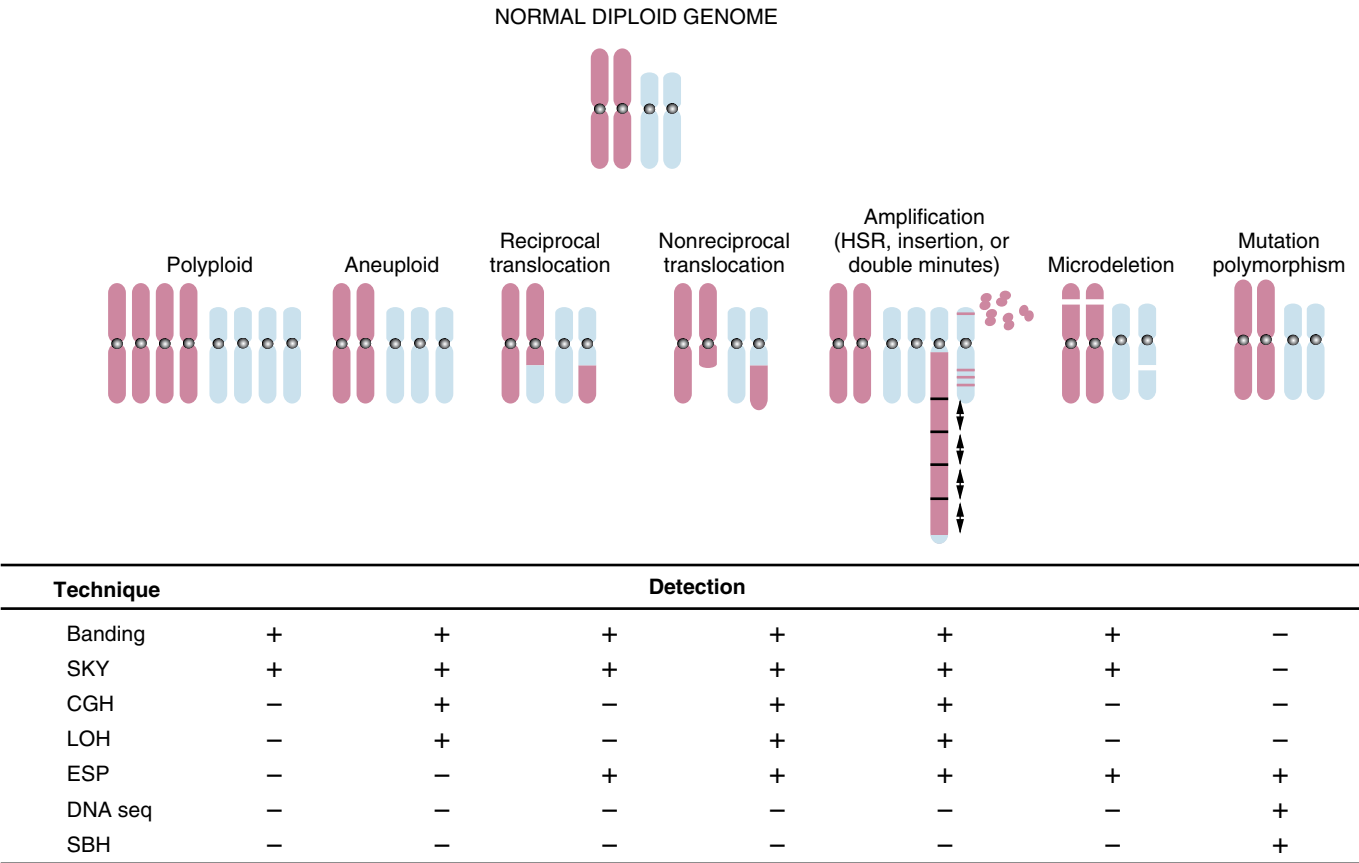


FIGURE 20-2 Schematic illustration of genome aberrations and detectability using common genome analysis techniques. (Adapted from Albertson DG, Collins C, McCormick F, et al. Chromosome aberrations in solid tumors. *Nat Genet* 2003;34:369-376, with permission.)

Table 20-1 Somatic Mutations Present in More than 5% of Designated Cancer Type, in Decreasing Frequency

Tumor Type	Gene	Tumor Type	Gene
Brain	<i>PTEN, CDKN2A, SMARCB1, PIK3CA, EGFR</i>	Ovary	<i>KRAS, BRAF, CTNNB1, CDKN2A, PIK3CA</i>
Breast	<i>PIK3CA, CDH1, TP53, CDKN2A, PTEN</i>	Pancreas	<i>KRAS, CDKN2A, MADH, CTNNB1, APC</i>
Large intestine	<i>KRAS, APC, BRAF, CTNNB1, PIK3CA</i>	Prostate	<i>KRAS, PTEN, HRAS, CTNNB1, BRAF</i>
Lung	<i>EGFR, KRAS, CDKN2A, TP53, RB1</i>	Skin	<i>BRAF, NRAS, CDKN2A, PTCH, PTEN</i>
Testis	<i>KIT, KRAS, NRAS, MADH4, STK10</i>	Urinary tract	<i>FGFR1, CDKN2A, HRAS, RB1, TP53</i>

*Source: <http://www.sanger.ac.uk/genetics/CGP/cosmic/>

type of mutated genes varies according to tumor type. Table 20-1, for example, summarizes somatic mutations reported in several common solid tumors (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>). The most frequently mutated genes vary according to tumor type or subtype. However, *CDKN2A*, *TP53*, *KRAS*, *BRAF*, *PIK3CA*, and *PTEN* are mutated at relatively high frequency in most solid tumor types. Importantly, comprehensive gene resequencing efforts suggest that somatic mutations occur in a much larger number of genes also play roles in cancer pathophysiologies (27,28). However, these events are typically found at frequencies below 1%, making it difficult to consider them individually interesting as either markers or therapeutic targets. These mutations may be best considered in ensemble as pathway modifiers and future interpretations may be made in this light.

The Epigenome

Epigenomic events such as DNA methylation or histone modifications control which regions of the genome are actively transcribed. Specific epigenomic events, illustrated in Figure 20-3, include methylation of a cytosine in regulatory CpG dinucleotides via DNA methyltransferases, DNA demethylation (29–31) and histone modifications (32,33) including phosphorylation, acetylation, methylation (mono-, di-, and trimethylation), ubiquitylation, ADP ribosylation, deimination, proline isomerization, and sumoylation (33). The histone modifications may directly alter protein–histone interactions or indirectly influence protein–histone or protein–DNA interactions by attracting other proteins that bind specifically to modified histones (34–38).

Epigenomic aberrations that contribute to cancer development often occur early during the process. These include aberrant hypermethylation of CpG islands in promoter regions associated with gene silencing (38–42) and genome-wide hypomethylation (43–48). Aberrant CpG island methylation has been assessed in several genes already known to play a role in tumor development. These analyses have identified aberrant methylation-mediated silencing of genes involved in most aspects of tumorigenesis, commonly altering the cell cycle (49–54), blocking apoptosis (55–59) or DNA repair (60–66). In general, aberrant CpG island methylation tends to be focal, affecting single genes, but not their neighbors (67,68). However, two loci have been found that exhibit epigenetic silencing over 150 kb in one case and 4 MB in the other (35,69,70). Other studies have found tumors that exhibit a global decrease in 5-methylcytosine relative to matching normal tissues (43,45,47,71–73). In severe cases, hypomethylation can affect more

than 10 million CpGs in a single tumor (74). Proposed mechanisms by which hypomethylation contributes to malignancy include transcriptional activation of oncogenes, loss of imprinting (LOI), and promotion of genomic instability via unmasking of repetitive elements (43,71). Disruption of histone modifications also are common in tumors. Both the pattern and the overall amount of each histone modification are important (38). For example, promoter histone H3 trimethylation of lysine 27 has been associated with gene silencing. These and other silencing marks may co-occur with aberrant DNA methylation and function synergistically in gene silencing or may act alone. Global changes in gene expression may result from the substantially decreased acetylation of Lys16 and trimethylation of Lys20 on histone H4, typically in repetitive portions of the genome and in association with hypomethylation of these DNA sequences (75). These large-scale alterations also may predict tumor recurrence in prostate cancer (76). Genomic sites of transitions between activating and repressive histone modifications also coincide roughly with common sites of translocations in human cancers, suggesting an additional association between these specific epigenetic states and chromosome instability in cancer (77).

Translational Applications

Cancer Risk and Early Detection

DNA-level abnormalities are appealing as markers for early cancer detection because of the relative stability of DNA in tissue sections and in peripheral blood, urine, sputum, and feces. Assays for genomic and epigenomic abnormalities already have proven useful for detection of early breast cancer lesions in histologically normal breast tissue (78) and for detection of bladder, lung, and colorectal cancer lesions in samples of urine (79–82), sputum (83–88), and feces (89–97), respectively. In addition, substantial efforts in early cancer detection are devoted to assays that detect aberrant DNA methylation in minute samples obtained with minimally invasive procedures such as sputum, blood, feces, urine, and nipple aspirates, and which likely contain tumor cells and tumor DNA shed from a primary tumor mass (98,99). In addition, loss of methylation from normally methylated promoters followed by gene activation elicit production of antibodies that are detectable in blood of patients with melanoma and other cancers (100).

Genomic and epigenomic aberrations also have been associated with cancer risk. Important examples of germ-line DNA

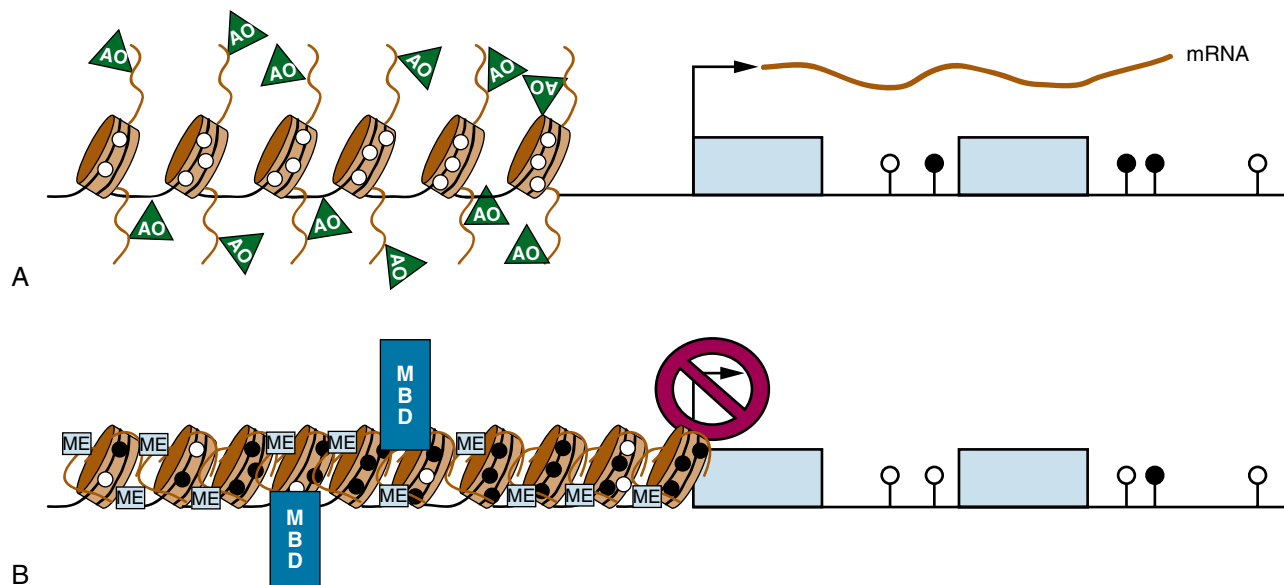


FIGURE 20-3 Schematic illustration of how epigenomic modifications affect gene expression. **A:** Open chromatin is comprised of nonmethylated DNA (open circles) and acetylated histones (AO). Transcription factors can assemble on this composition and initiate RNA polymerase mediated transcription. **B:** Closed chromatin resulting from methylation of DNA (Filled circles) by DNA methyltransferases and histone deacetylation resulting from recruitment of methyl-binding domain proteins (MBD) and associated histone deacetylases. Closed chromatin is inaccessible to transcription factors and transcription is inhibited.

mutations that substantially increase cancer risk include mutations in *RB1* causing childhood retinoblastoma (22), *TP53* associated with the Li-Fraumeni cancer syndrome (101), *BRCA1* and *BRCA2* leading to early-onset breast and ovarian cancer (102), *MLH1* and other DNA-repair genes associated with hereditary nonpolyposis colon cancer (103), and *CDKN2A* associated with familial atypical multiple mole melanoma and pancreatic cancer (104). Epigenetic gene inactivation of these same genes, particularly *MLH1*, *MSH2*, and potentially *DAPK1* also may contribute to increased cancer risk (59,105–107).

Evidence is emerging, especially from mouse model studies, that highly prevalent germ-line polymorphisms exist that modestly increase susceptibility to cancer individually but may combine to dramatically increase individual cancer risk (108). Interestingly, genetic polymorphisms associated with increased or decreased cancer susceptibility may be selectively amplified or deleted, respectively during cancer progression so that combined analyses of genotype and copy number may facilitate their discovery (109).

Predictive/Prognostic Markers

High-level amplification has been associated with poor outcome in numerous published studies. Well-known examples include the association of (1) *NMYC* amplification with reduced survival duration in neuroblastoma (110), (2) amplification of *HER2* with reduced survival duration in breast cancer (9), (3) mutation of *EGFR* has been associated with response to Iressa (111), (4) amplification of the *BCR/ABL* gene fusion in Gleevec-resistant tumors (112), and (5) amplification of *AR* has been associated with the development of androgen independence in prostate cancers (13). High-resolution CGH analyses have revealed many other genome copy number aberrations that are associated with altered survival

duration. Many of the studies establishing associations with outcome are small and in most cases, the responsible genes have not been definitively identified. The importance of these associations will clarify with time. Specific mutations also have been associated with reduced survival duration. The most prominent example is *TP53* (113).

Aberrant methylation of particular CpG islands may also alter the response of a cancer cell to therapeutic agents or serve as a clinically useful marker of clinical outcome (114–117). For example, normal expression of the DNA repair gene, O-6-methylguanine DNA methyltransferase (*MGMT*), is associated with resistance to therapy, whereas aberrant methylation of the *MGMT* 5' CpG island, and presumably *MGMT* silencing (60,61,118), is associated with significantly improved antitumor response of alkylating agents such as Temozolomide (64,119). In contrast, cisplatin-resistant cancer cells can be sensitized by relieving repressive histone H3 K27 methylation and DNA methylation, presumably by reactivating silenced tumor suppressors and modulators of cisplatin response (120). A degree of loss of imprinting at *IGF2* can be detected in a subset of normal individuals, clusters within families, and may be transmitted transgenerationally; its detection in blood cells may be a predictive marker for an individual's risk of colorectal cancer (121–124).

Therapeutic Targets

Numerous genomic and epigenomic somatic aberrations have been associated with tumor outcome in recent years and several are now being developed as therapeutic targets. U.S. Food and Drug (FDA)-approved targeted therapies include trastuzumab and lapatinib, targeting amplified *ErbB2*; Imatinib mesylate targeting the *BRC-ABL* fusion gene; and Gefitinib targeting tumors

with *EGFR* mutations. Epigenomic abnormalities also are being targeted therapeutically. Examples of drugs that have shown some clinical efficacy include DNA methyltransferase inhibitors such as 5-azacytidine (Vidaza), 5-aza-2'-deoxycytidine (Decitabine/Dacogen), RG108, and an antisense oligodeoxynucleotide directed against the 3' untranslated region of the DNA methyltransferase-1 enzyme mRNA designated MG98, and histone deacetylase (HDAC) inhibitors such as butyrate, depsipeptide, suberoylanilide hydroxamic acid (SAHA, vorinostat; 125,126). There are more drugs on the way. For 2006, the Pharmaceutical Research and Manufacturers of America (<http://newmeds.phrma.org/>) reported that 646 medicines were under development for cancer. Approximately 400 of these are already in phase 2 or phase 3 trials and most are gene or pathway targeted.

Integrative Analyses

A major challenge for the future is to understand how genomic and epigenomic aberrations cooperate directly or indirectly (68,127) to develop the pathophysiologies that define human malignancies. Several possibilities exist. For example, genomic and epigenomic aberrations may cooperate directly to complete inactivation of tumor suppressors or by methylation of one allele and deletion or mutation of the other (128,129). Examples include the transcription factor 21 (*TCF21*) in head and neck and lung cancers (70) and oligodendrocyte transcription factor 1 (*OLIG1*) in lung cancer loss (114,130). Other putative tumor-suppressor genes located in regions of frequent LOH, such as *DLEC1*, *PAX7*, *PAX9*, *HOXB13*, and *HOXB1*, have been identified via the use of affinity columns to enrich methylated DNA sequences (131). Given their specific technical limitations, these studies indicate that the integration of several experimental strategies will be required to maximize the discovery of new cancer-related genes.

Interestingly, evidence is also emerging that genomic and epigenomic aberrations play roles at different stages of tumor development. In breast cancer, for example, epigenomic aberrations seem to predominate in early phases of the disease with genomic aberrations becoming important later in the process (132,133). This may be due to the fact that epigenetic mechanisms can cause genomic alterations. For example, aberrant methylation-associated silencing of *MLH1* leads to microsatellite instability in colon cancer (65,66) whereas methylation and silencing of the cell-cycle checkpoint gene *CDKN2A* have been associated with aberrant centrosome function and genome instability (134). However, genomic aberrations also can influence the epigenome. For example, translocations of *PML* and *RARA* can create a fusion protein that abnormally recruits the DNA methyltransferase and may cause aberrant methylation at specific promoters in leukemia (135) whereas global CpG island methylation (136,137) has been associated with genetic mutations of *BRAF*. In general, integrative analyses of the types suggested previously will be required if we are to achieve a complete understanding of the molecular events that contribute to tumorigenesis and progression.

Analysis Technologies

Our ability to define the genomic and epigenomic events that contribute to cancer pathophysiology and response to therapy is determined by the analytical technologies that can be used to discover them. The power and genomic precision of analytical approaches are increasing dramatically as the technologies and information from the human genome project are harnessed for these purposes. Representative technologies for analysis of genomes and epigenomes are summarized in the following sections.

Genome Analysis Techniques

The earliest cancer-associated recurrent genome aberrations were discovered via analysis of metaphase chromosomes following banding analysis to discriminate between chromosome types, although now fluorescence in situ hybridization (FISH) is added as a complement to banding for metaphase chromosome discrimination. Cytogenetic techniques have been particularly useful for analysis of the comparatively "simple" cancer genotypes of leukemias and lymphomas but have been difficult to apply to the more complex cancer genotypes associated with solid tumors. These genomes have been more successfully analyzed using genome wide techniques such as comparative genome hybridization (CGH) that map changes onto the normal representation of the human genome to facilitate interpretation. More recently, high throughput genome sequencing has been added to the armamentarium of genome analysis tools that are being brought to bear on tumor genomes. These techniques are reviewed in the following sections.

Cytogenetics

Cytogenetic studies begin with the production of chromosome preparations in which metaphase cells are swollen hypotonically and mechanically ruptured so that the chromosomes become spread over small regions of a microscope slide. The chromosomes are then stained so that the chromosomes can be individually recognized and rearrangements identified and classified. Q-banding using alkylating fluorochromes was introduced for this purpose by Caspersson and colleagues in 1970 (138). This allowed individual chromosomes and aberrations therein to be identified with high accuracy. This was followed by a large number of different banding chemistries. An informative chronology of the various banding techniques can be found at <http://homepage.mac.com/wildlifeweb/cyto/text/BandingHistory.html>. Modern banding techniques generate from 300 to more than 850 bands on the 24 chromosomes types. An International System for Human Cytogenetic Nomenclature was established by expert committees to standardize chromosome classification using banding analysis (139,140). A compendium of chromosomal abnormalities discovered through analysis of human malignancies compiled by Mitelman and colleagues is available at <http://cgap.nci.nih.gov/Chromosomes/Mitelman>.

Although banding analysis is a powerful chromosome classification technology, it suffers from three limitations: (1) It requires substantial training in order to learn to interpret the patterns

accurately; (2) banded metaphase preparations often cannot be interpreted for solid tumors with complex “shattered” genomes; (3) preparation of high-quality, well-banded metaphase spreads is sometimes difficult or impossible from solid tumors.

Fluorescence In Situ Hybridization (FISH)

FISH is a technique that allows specific genome regions to be visualized in the light microscope, either in metaphase spreads or in interphase nuclei where the chromosomes are not condensed into discrete bodies (141–145). FISH is based on the concept that specific DNA sequences can be “stained” in cytologic context by reacting fluorescently labeled, single-stranded nucleic acid “probes” with cellular preparations in which the DNA has been made single stranded—typically by heating so the hydrogen bonds that normally maintain the double helix are broken. The probe–cytologic preparations are then cooled to allow the probes to bind (hybridize) to the sequences to which they are homologous. Generally, unlabeled repetitive DNA fractions are added during hybridization to competitively suppress hybridization of labeled repetitive sequences that are present in most genomic probes. The preparations are then washed to remove the unbound probe and visualized using fluorescence microscopy. FISH is used routinely for detection of a broad range of genome copy number and structural aberrations in metaphase preparations and interphase nuclei. The process and typical results are illustrated in Figure 20-4. FDA-approved assays include a test for *Her2* amplification in breast cancer (146) and a multitarget test for aneusomy involving chromosomal aneuploidy at chromosomes 3, 7, and 17 and loss at 9p21 as a marker for bladder cancer (147).

Comparative Genomic Hybridization

CGH allows changes in genome copy number to be mapped onto a representation of the normal genome thereby allowing ready identification of the genes involved in the aberrations (148). In early CGH analyses, DNA samples from tumor and normal genomes were labeled with different fluorochromes and hybridized to metaphase chromosome spreads (148). Since the rate of hybridization is concentration dependent, the ratio of the intensity of the “tumor fluorochrome”

to the intensity of the “normal fluorochrome” was measured as an estimate of the relative tumor to normal genome copy number at each location along the chromosomes to which they were hybridized. The resolution of chromosome-based CGH is limited to about 10Mbp by the nonlinear packaging of DNA along the chromosomes. This resolution limitation has been removed by replacing metaphase chromosomes with arrays of bacterial artificial chromosomes (BACs), cDNA, or oligonucleotide (149,150) “probes” as the genome representations onto which genome copy number changes are mapped so that subgene resolution can be achieved readily. This procedure and typical results are illustrated schematically in Figure 20-5.

CGH has been used worldwide to identify genome copy number changes in a broad range of human tumors. The major advantage of the technique is that it maps changes in genome copy number onto a normal representation of the genome so it is straightforward to identify the genes that are involved in recurrent copy number aberrations. In addition, it gives semiquantitative information about level of gain or loss so it is possible to distinguish, for example, between high-level amplification and gain of a single copy of a chromosome and to distinguish between loss of one chromosome or region and homozygous loss of a segment of the genome. Some techniques allow copy number to be measured in an allele-specific manner (151) so it is possible to identify loss of one allele and duplication of the remaining allele and to survey for allele specific amplification—events that might indicate selection for or against germ-line polymorphisms that are associated with cancer susceptibility or resistance, respectively. Recurrent genome copy number abnormalities discovered using CGH completed by Knuutila and colleagues can be found at http://www.helsinki.fi/cm/cgh_data_1.html.

DNA Sequence Abnormalities

Mutations that participate in the oncogenic process may be found in the coding sequences, at splice sites, or in DNA sequences that regulate transcription. Several technologies for discovering and validating these aberrations, including established and representative new approaches, are summarized in this section.

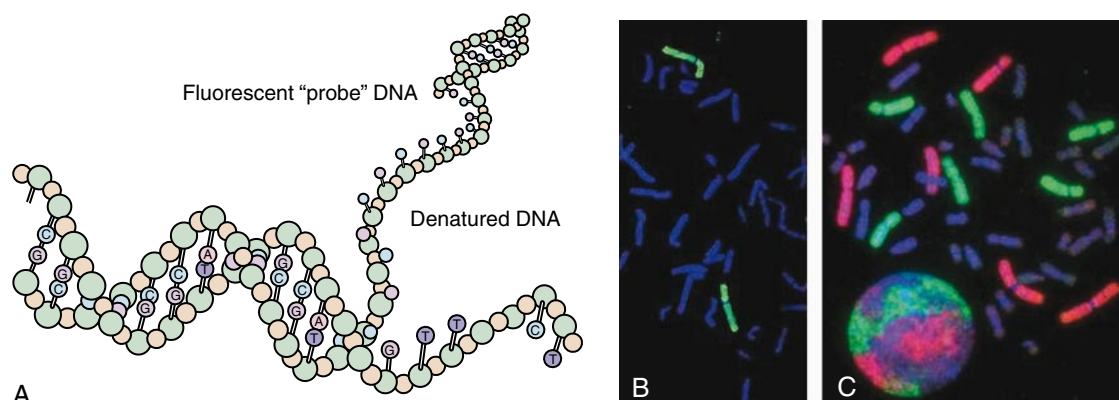


FIGURE 20-4 FLUORESCENCE IN SITU HYBRIDIZATION (FISH) CONCEPTS AND RESULTS. **A:** Schematic illustration of the in situ hybridization process in which fluorescently labeled DNA is used to label specific DNA sequences “in situ.” **B:** Hybridization with a chromosome specific probe to a metaphase spread. Hybridized probe fluoresces green. DNA is counterstained with a blue fluorescing dye. **C:** Hybridization to metaphase and interphase nuclei using chromosome specific probes labeled with fluorescent molecules that emit at different wavelengths. Individual chromosome domains are apparent in metaphase and interphase nuclei.

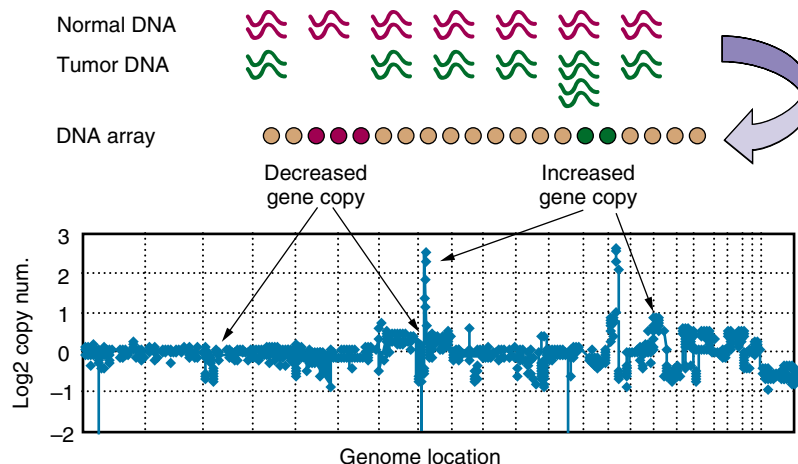


FIGURE 20-5 COMPARATIVE GENOMIC HYBRIDIZATION (CGH) CONCEPTS AND RESULTS. The principles of CGH are illustrated in the upper part of the figure. In one embodiment of CGH, differentially labeled normal (red) and tumor (green) samples are hybridized to a representation of the normal genome (an array of DNA fragments in this example). The lower panel shows a CGH analysis of a typical human breast tumor. Copy number increases present as values greater than zero while decreases present as negative numbers after logarithmic transformation. Data are displayed as a function of distance along the genome with chromosome 1 to the left and chromosomes 22 and X to the right. The vertical lines indicate chromosome boundaries.

Sequencing by Hybridization

Sequencing by hybridization (SBH; 152) is similar to CGH but is designed to detect mutations, taking advantage of the fact that nucleic acid hybridization conditions can be developed so that the intensity of hybridization of a target nucleic acid to a short oligonucleotide is significantly higher when the probe sequence is perfectly complementary to the target than when a single-base mismatch exists. In this approach, test samples are hybridized to arrays comprising short oligonucleotide probes that are designed to be perfectly complementary to the reference sequence plus oligonucleotide probes that differ by one base at each “substitution position” in the genome to be tested for mutation. Each of the four possible nucleotides is represented in the probe set at each substitution position. Extended regions of the genome can be sequenced using this approach since it is possible to manufacture microarrays or microbead platforms carrying millions of oligonucleotides. This approach is well suited to resequencing of regions of the genome known to be frequently mutated or to encode frequent single-nucleotide polymorphisms (SNPs). For example, commercial assays are available that interrogate SNPs at about 1 million loci in human samples or that can be used to resequence important regions including TP53 and the 16 Kbp comprising the entire human mitochondrial genome. It is not as well suited to large-scale genome analyses, although this has been accomplished in some commercial settings. In addition, it is not well suited to detection of mutations that occur at low frequency in a test sample.

Dideoxy Sequencing

Dideoxy sequencing (chain termination or Sanger sequencing; 153) is the workhorse of traditional mutation detection because the methodology is robust and approved for clinical applications. The technology and illustrative results are presented in Figure 20-6. The first step of dideoxy sequencing is to create a purified population of DNA molecules that are to be sequenced. For mutation detection, this DNA population would be the product of a PCR reaction. The double-stranded DNA is melted into separate strands and a primer complementary to the 5' end of one of the

strands is annealed. Nucleotides and DNA polymerase are added and the mixture is then split into four separate extension reactions each of which receives a separate dideoxynucleotide (which acts as a chain terminator upon addition) and fluorescent label (154). The result of these reactions is a set of molecules of different lengths where the length of the molecule is tied directly to its fluorescent emission. These mixtures are subjected to electrophoresis in a capillary, which moves the DNA molecules past a detector by size, the smallest molecules pass the detector first, producing a map of fluorescent color versus length. These chromatograms can be deconvolved into the nucleotide sequence of the originating molecules. Sequence “reads” typically are about 750 bp.

When dideoxy sequencing PCR products, representing a specific genomic location that is heterozygous at a given base position, the chromatograms will show a position in which two bases are present because the originating sample will be a mixture of the two molecules. Similarly, a sample that carries an insertion or deletion (indel) will begin to show two sequences that overlap and are offset from one another at the nucleotide position where the indel begins by the number of base pairs that have been inserted or deleted (Figure 20-7). It is important to realize that most implementations of mutation detection using dideoxy sequencing will miss mutations that are present in a small fraction of the cells in the PCR-amplified population. Consider the common case of a sample that is only 30% tumor (a low but unfortunately realistic number) and a mutation that is only present in 20% of the tumor. Only 3% of the DNA molecules in that sample would harbor a mutation if it were heterozygous. Nearly all dideoxy sequencing systems will fail to detect this since the minor allele must be at least 20% of the major allele to be confident of a mutation's presence. Likewise, mutations present in a few percent of tumor cells will be missed, even in a sample that is entirely tumor.

Dideoxy sequencing is being used by international efforts to identify mutations in cancer genomes. Several studies have reported genome-wide efforts to identify cancer specific mutations through analysis of cancer cell lines and primary tumors. These studies point to the existence of hundreds of causal mutations. However, most of these are present in no more than 5% of the tumors of any given type. Mutations discovered and estimates of their frequencies

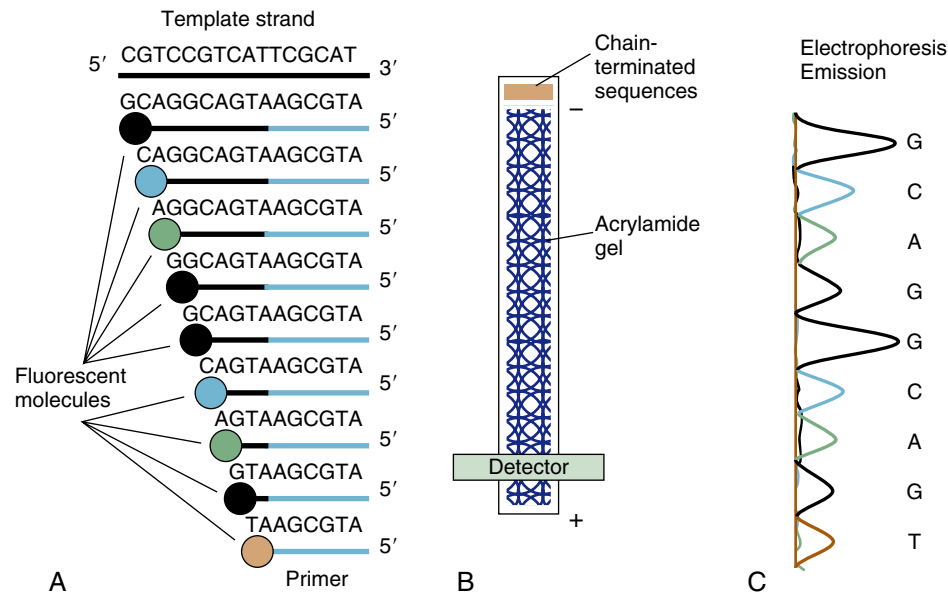


FIGURE 20-6 DNA SEQUENCING USING CAPILLARY DNA SEQUENCERS AND FLUORESCENT CHAIN TERMINATORS. **A:** Template DNA is mixed with a mixture of primer, polymerase, nucleotides (dATP, dTTP, dCTP, dGTP), and differentially fluorescently labeled chain terminators (ddATP, ddTTP, ddCTP, ddGTP). Chain-terminating nucleotides are present at a much lower concentration than normal nucleotides. The primer is a short DNA molecule (≈ 20 bp) complementary to the 3' end of the template strand. The primer is allowed to hybridize to the template strand, creating a partial duplex sequence that provides the polymerase enzyme with the starting point for DNA synthesis. The polymerase then incorporates individual nucleotides complementary to the template sequence at the 3' end of the growing sequence. If a chain-terminating nucleotide is incorporated, no additional bases can be added and a DNA chain of a fixed length is created. **B:** Chain-terminated sequences are loaded into one end of a capillary filled with an acrylamide gel. The gel in the capillary is porous allowing smaller molecules to move through it more easily than larger molecules. In the presence of an electric field, DNA, which is negative charge, migrates toward the positive electrode, which is placed on the opposite end of the capillary from the side that is loaded. Prior to reaching the end of the capillary, the molecules pass in front of a fluorescent detector, which records the fluorescence as a function of molecule size. **C:** Fluorescent spectra are deconvoluted and the DNA sequence is identified using statistical methods that predict the base and the likelihood of misassignment at each position. Quality scores (calculated as the negative logarithm of the p value confidence) are calculated for each base in the DNA sequence.

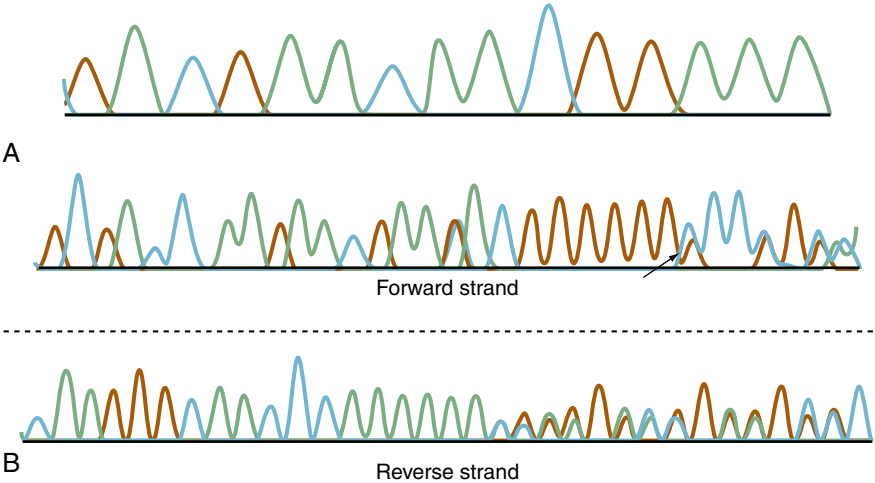
of specific tumors are summarized at a site curated by the Wellcome Trust Sanger Institute and can be found at <http://www.sanger.ac.uk/genetics/CGP/cosmic/>.

Whole-Genome Paired-End Shotgun Sequencing

Successful sequencing of the human genome and genomes of many model organisms has been accomplished using whole genome sequencing strategies (155,156). In “shotgun sequencing” a target genome is fragmented randomly into numerous small segments, which are sequenced using dideoxy sequencing. This requires that most regions of the genome will be sequenced many times (typically

10 to 12 times) to ensure that all regions of the genome are covered adequately. Computer programs assemble overlapping sequences into a contiguous sequence. This assembly is complicated by the fact that the human genome has repetitive sequences interspersed throughout, so that sequences carrying the same (or very similar sequences) may be improperly joined at the repeat. To facilitate assembly, large insert clones (e.g., 10, 50, and 150 Kbp) can be prepared, end sequenced, and then individually shotgun sequenced and assembled. The small size of each clone makes local assembly straightforward. These local assemblies can then be joined with the shotgun sequences and assembled into scaffolds as illustrated in Figure 20-8.

FIGURE 20-7 EXAMPLES OF MUTATIONS IN DNA SEQUENCES. **A:** DNA sequence trace showing a substitution mutation. The seventh base pair from the left shows two peaks an A and a C where the normal DNA sequence shows only an A. The height of the heterozygous base is lower for the A allele than the A bases on either side. **B:** DNA sequence trace showing a 1-bp deletion. One of the T bases has been deleted between positions 20 and 26 in the forward strand in the mutant chromosome copy. The DNA sequence past the deletion becomes very difficult to interpret. The reverse strand shows the sequence observed in the other direction.



END SEQUENCES

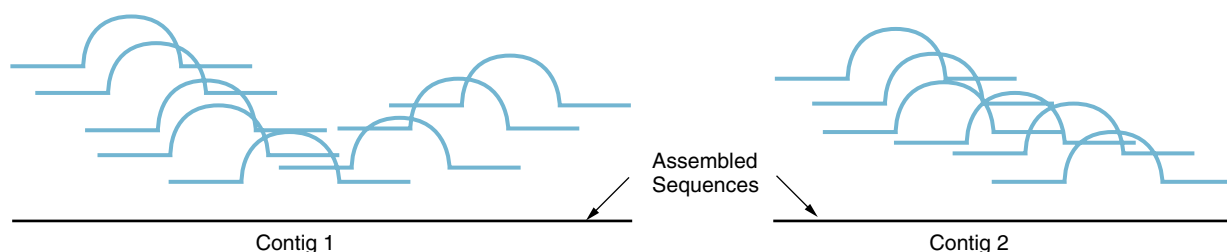
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5' CAAGGGGGGAG ---Unknown Insert DNA Sequence--- AGGAGGAGTGGG 3'
5' TGCTGCGAGGG ---Unknown Insert DNA Sequence--- GAAGGAAAAGGG 3'
5' AGGCAGCAGAG ---Unknown Insert DNA Sequence--- CCGGGCAGAGTC 3'
5' CGAACCACAG ---Unknown Insert DNA Sequence--- CCAGAAGCCCGC 3'
5' ACGCACCTCGC ---Unknown Insert DNA Sequence--- ACCATGAGATGG 3'
5' CGACGCGCGGC ---Unknown Insert DNA Sequence--- CCAGCGCCCCGG 3'

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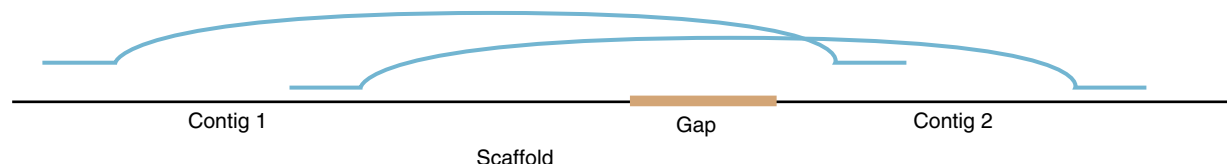
A

CONTIG CREATION



B

SCAFFOLD CREATION



C

FIGURE 20-8 SCHEMATIC ILLUSTRATION OF A SHOTGUN DNA SEQUENCING ASSEMBLY PROCESS. **A:** Generation of DNA sequences from paired end reads. Three separate random DNA sequence libraries are created with three different sizes of inserts (small, $\approx 2,000$ bp; medium, $\approx 10,000$ bp; and large, $\approx 50,000$ bp). Smaller libraries are more robust (better insert size control and better sequence reads) but provide less coverage of the genome. Enough DNA sequences are generated to sequence the entire genome approximately ten times. **B:** Illustration of the creation of contigs. Repetitive sequences are masked. Paired end reads for the small insert for which both sequences align are joined into contigs. **C:** Larger insert paired end reads are used to link contigs together.

End-sequence profiling (ESP) is an adaptation of whole-genome shotgun sequencing that facilitates detection and DNA sequence analysis of structural aberrations including translocations, inversions, and segmental deletions that are not easily detected using other DNA sequence analysis technologies (157) but does not identify small mutations. In this process, DNA from the tumor of interest is cloned into BACs or other relatively large insert vectors and the ends of the resulting clones are sequenced and mapped onto the normal human DNA sequence as illustrated schematically in Figure 20-9. Paired ends that map farther apart than the maximum size tolerated by the cloning vector (e.g., about 150 Kbp for BACs) indicate that the clone contains a segment of the genome carrying a structural aberration, deletion, or chromosome fusion. These clones can then be sequenced using strategies described previously to identify the involved genes.

Single-Molecule Sequencing Methods

Technologies have been commercialized that allow genome-wide DNA sequencing starting from single molecules rather than a population of molecules. The methods each have their own unique features but they fundamentally change the process of mutation detection because they are massively parallel. Whereas the most efficient dideoxy sequencers generate sequences from a few thousand templates in a day, these new technologies generate sequences

from 100s of thousands to tens of millions of templates in a day (158,159). Several different approaches have been commercialized but most operate according to the same principle. One example of the single-molecule technologies is the technology from Illumina/

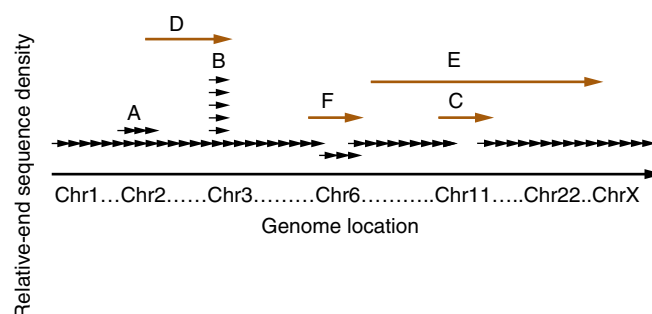


FIGURE 20-9 SCHEMATIC ILLUSTRATION OF THE RESULTS OF END-SEQUENCE PROFILING (ESP). In ESP, a tumor genome is cloned into a large insert vector such as a bacterial artificial chromosome (BAC) and both ends of several thousand clones are sequenced and mapped onto the normal genome. The density of end sequences is a measure of relative tumor genome copy number. Clones whose ends appear to be the right distance apart are shown as black arrows. Clones whose ends appear too far apart are shown as red arrows. **A:** A segmental copy number increase. **B:** Regions of high-level amplification. **C:** Regions of homozygous deletion and the existence of a clone that spans the deletion. **D:** Existence of a clone that joins an amplified sequence near chromosome 3 to a segmental duplication on chromosome 2. **E:** Existence of a clone that carries sequences that map to different chromosomes such as might result from a translocation. **F:** A segmental deletion and the existence of a clone that carries sequences from both sides of the deletion.

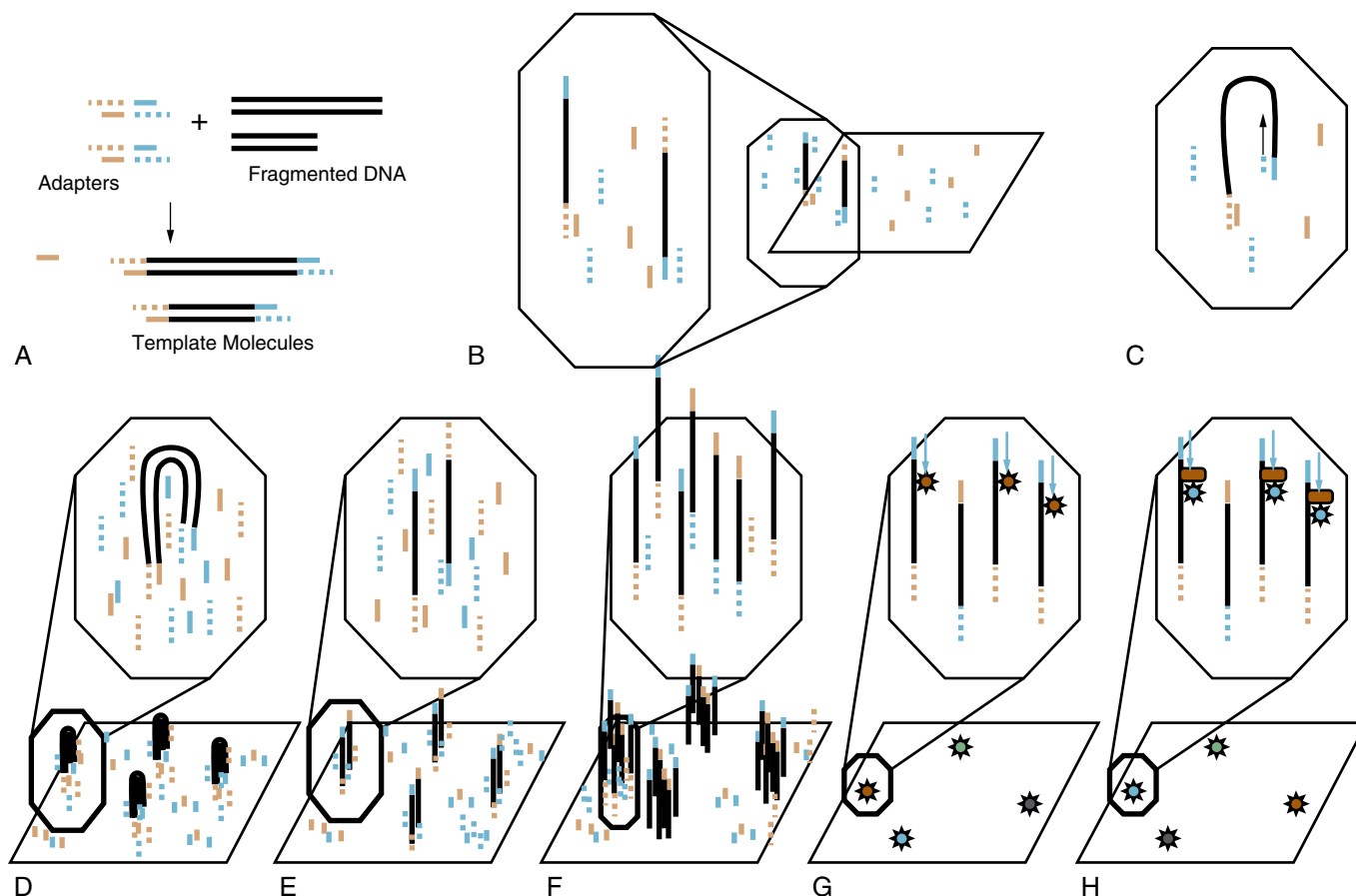


FIGURE 20-10 Schematic illustration of single-molecule sequencing as implemented by Solexa/Illumina. Slides are adapted from <http://www.illumina.com/>. Sequencing is accomplished in several steps. **A:** DNA is fragmented and ligated to adapter DNA fragments. **B:** The fragments with adapters are denatured and captured on a dense lawn of oligonucleotides homologous to the adapter molecules. **C, D:** DNA fragments captured on both ends (bridged) are amplified using primers to the adapter sequences. **E, F:** The denaturation and bridge amplification steps are repeated several times to produce a collection of identical molecules (a polony) at each site of initial capture. **G, H:** The polonies are sequenced in parallel using a cyclic process. In the first step, primers, a polymerase and four reversibly 3-terminated bases (A, T, G, C) labeled with distinctive fluorochromes are added and the surface is washed. The entities of the bases added at the first step are determined for each polony using fluorescence microscopy. Next, the 3' terminators and fluorochromes are removed, new fluorescently labeled, 3' terminated bases are added with polymerase and the identities of the newly added bases are determined for each fluorochrome as before. This cycle is repeated several dozen times to generate DNA sequence information for each polony.

Solexa (<http://www.illumina.com/>), which is shown conceptually in Figure 20-10. In this approach, single molecules are captured on a substrate and amplified at that site using strategies that capture the amplified sequences so they remain locally confined. The sequences of the localized DNA fragments are then determined in parallel. The reaction has been engineered so that a light signal is generated each time a base is added. Similar approaches have been implemented by 454 Life Sciences (<http://www.454.com/enabling-technology/the-process.asp>) and Applied Biosystems (http://marketing.appliedbiosystems.com/images/Product/Solid_Knowledge/SOLiD_Chemistry_Presentation_1019.pdf).

Single-molecule sequencing promises to impact mutation detection in numerous ways. First, the technologies dramatically lower the cost of DNA sequencing by parallelizing every step of the assay. The expectation is that costs per base using these technologies will be 100 to 1,000 times less expensive than the current dideoxy sequencing methods. Second, these technologies make it practical to identify rare mutations. Presently, to find rare mutations it is necessary to clone individual molecules and sequence each one. This requires that sufficient clones be sequenced to find

the rare variants. Using dideoxy sequencing identifying mutations in this way costs at least 100 times more than detecting mutations that are prevalent because hundreds of molecules must be sequenced at each locus to be tested. One caveat to single-molecule sequencing is that rare errors can be introduced into the DNA sequence during the process so an added level of validation is necessary.

Single-molecule sequencing does not solve the problem of determining which gene regions to sequence. It is still impractical to resequence the entire genome so individual PCR fragments or some other selection scheme is necessary. The application of these methods is still in its infancy but it is expected to make a substantial impact on mutation detection as costs continue to decline and production capabilities continue to increase.

Epigenome Analysis Techniques

Techniques for analysis of the epigenome have evolved rapidly from single gene approaches to genome wide techniques for assessment of DNA methylation and chromatic structure. Both

single-molecule and microarray techniques are now employed. Selected techniques are summarized in the following sections.

DNA Methylation

Several strategies have now been developed to assess methylation at CpG islands that influence gene expression. These include PCR strategies to assess methylation at single genes, and genome scanning technologies based on large-scale restriction fragment analysis, microarray approaches and genome wide sequencing.

Methylation-Specific Polymerase Chain Reaction

Mapping of DNA methylation patterns in CpG dinucleotides in specific genes can be accomplished using methylation-specific PCR (MSP). In this approach, the test DNA is first modified by treatment with sodium bisulfite, which converts unmethylated, but not methylated, cytosines to uracil. The modified DNA is then amplified with two sets of primers, one that is specific for the unmethylated template and one that is specific for the methylated template. MSP requires small quantities of DNA, is sufficiently sensitive to detect 0.1% methylated alleles at any locus, and can be performed on DNA extracted from paraffin-embedded samples. This approach is well suited to analysis of methylation at specific CpG sites. However, it is not generally used for genome-wide methylation discovery efforts or for efforts requiring quantitation of methylation levels.

Restriction-Landmark Genomic Scanning

Restriction-landmark genomic scanning (RLGS) was the first method developed as a genome-wide screen for CpG island methylation (160,161). In RLGS, illustrated schematically in Figure 20-11, genomic DNA is digested with a rare-cutting methylation-sensitive restriction enzyme such as *NotI* or *AscI* whose recognition sequences occur preferentially in gene associated CpG islands (162,163). These enzymes do not cut when CpG sequences targeted by the rare-cutting enzyme are methylated. Following digestion, the DNA is radiolabeled and subjected to two-dimensional gel electrophoresis. DNA methylation is detected as the absence of a radiolabeled

fragment, which occurs when the enzymes fail to digest a methylated DNA substrate. RLGS permits quantitative representation of methylation levels and has a low false-positive rate relative to most other global methods for detecting DNA methylation. Additionally, a priori knowledge of sequence is not required (164), making RLGS an excellent discovery tool (165–168). However, RLGS is limited to the number of rare-cutting methylation-sensitive restriction enzyme sites in the human genome that fall within DNA fragments that can be resolved well; typically about 4,000 when using a combination of *NotI* and *AscI* enzymes (161,169).

Microarray Epigenome Analysis

Microarray based methods for genome wide methylation analysis (170–177) rely on information on genome structure from the Human Genome Project (156,178). Differential methylation hybridization was the first array method developed for genome-wide CpG methylation analysis (176). In this assay, tumor and reference DNA samples are first digested with *MseI*, an enzyme that cuts preferentially outside of CpG islands, and then ligated to linker primers. The ligated DNAs are then digested with methylation-sensitive four-base restriction enzymes, such as *BstUI*, *HhaI*, or *HpaII*, that cut preferentially in GC-rich genomic regions, including CpG islands. The resulting tumor and reference DNA fragments are amplified by PCR using the ligated linkers as primer binding sites, differentially labeled with distinctive fluorochromes and hybridized to microarrays carrying oligonucleotide probes for candidate CpG islands. DNA fragments that are methylated in the tumor samples will not be cut with the methylation-sensitive restriction endonucleases and so will generate PCR products that will hybridize to cognate microarray probes while unmethylated sequences in the normal sample will not be cut or amplified and so will not hybridize to the microarrays. Thus, comparison of signal intensities derived from the tumor and reference samples following hybridization to CpG island arrays provides a profile of sequences that are methylated in one sample and not the other (171–175,179). Improvements in oligonucleotide arrays, particularly for allelic methylation analysis, hold

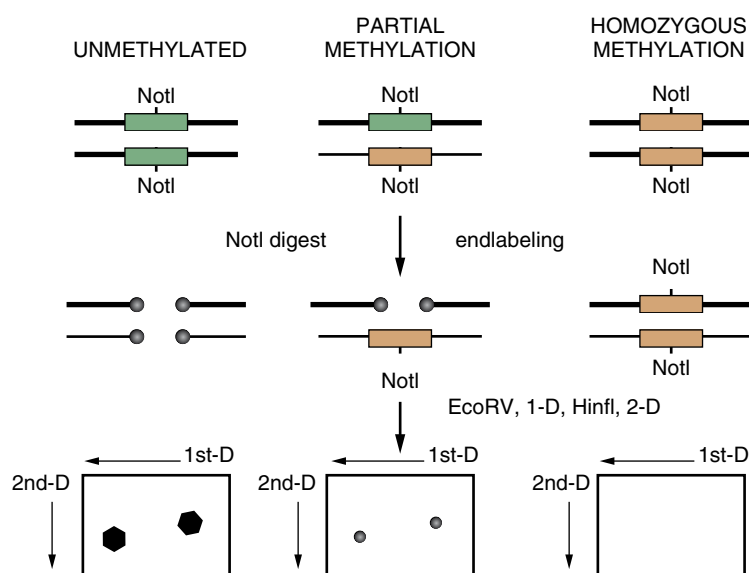


FIGURE 20-11 Schematic illustration of restriction landmark genome scanning (RLGS) showing the quantitative nature of methylation detection on *NotI* fragments displayed on RLGS profiles. Methylation detection in RLGS profiles depends on the methylation sensitivity of the endonuclease activity of *NotI* or other methylation sensitive restriction enzymes. Differences in digestion are assessed by radiolabeling the DNA at cleaved *NotI* sites. Following further endonuclease digestion, two-dimensional electrophoretic separation and autoradiography, the intensity of a DNA fragment on the resultant RLGS profile quantitatively reflects the copy number and methylation status of the *NotI* fragment. A priori, this allows *NotI* fragments containing single-copy CpG islands to be distinguished from the abundant *NotI* fragments present in repeat elements and rDNA sequences.

promise to bring even greater methylome coverage to methylation array-based methods in the future (171–175).

BAC arrays also have been successfully used for large-scale DNA methylation analysis (170,171,174,175). In this approach, tumor and reference genomic DNA samples are digested with a rare-cutting methylation-sensitive restriction enzyme that cuts preferentially in CpG islands, the digested sites are filled in with biotin, and unmethylated fragments are selected on streptavidin beads. The tumor and reference samples are then labeled with distinctive fluorescence molecules and cohybridized to a BAC array. Most BACs will contain only a single rare cutting methylation sensitive restriction enzyme site or single cluster of these restriction sites so that single CpG site resolution is achieved. As a result, the presence of an increased tumor-to-normal hybridization ratio at a BAC probe indicates methylation at the CpG within the restriction site that maps to this BAC. This methylation analysis is particularly useful for genome-wide assessment of CpG methylation using BAC arrays that tile contiguously across the entire genome.

The particular combination of array and methylation-sensitive detection reagent is also critical for tumor methylome analysis. These reagents include methylation-sensitive restriction enzymes, 5-methylcytosine antibody, methylated DNA-binding protein columns, or bisulfite-based methylation detection. Bisulfite is a chemical that allows conversion of cytosine to uracil, but leaves 5-methylcytosine unconverted (180). This method is a staple of single-gene analysis and high-throughput analysis of small sets of genes (181,182). However, due to the significantly reduced sequence complexity of DNA after bisulfite treatment, its use for array application is more limited (183,184). DNA selected through methyl-binding protein columns or by 5-methylcytosine antibody-immunoprecipitation has also been applied to microarrays (69,131,174,185–187). The effective resolution of methylation using either method is dependent in part on the average DNA fragment size after random shearing, generally 500bp to 1kb. It is not yet clear how many methylated CpG residues are needed for productive methylated DNA-antibody binding to occur or whether the antibody has significant sequence bias. An advantage of this approach is that it is not as limited to specific sequences as restriction enzyme-based approaches. However, the large amount of DNA required for this method currently may preclude its use for DNA extracted from archival cancer specimens. Whole-genome amplification after immunoprecipitation could circumvent this limitation, albeit with greater potential for sequence-representation bias. The 5-methylcytosine antibody approach has been used to map the methylome of *Arabidopsis thaliana* (185,187) and human cancer cell lines (69,186). Antibody and methylated DNA-binding columns with single-molecule sequencing also hold great promise (188).

Reduced-Representation Bisulfite Sequencing

Reduced-representation bisulfite sequencing (RBBS) is a large-scale, genome-wide shotgun sequencing approach (188) that will likely be applicable to assessment of aberrant methylation in tumors. In this approach, tumor and reference DNA samples are digested with BglII, size selected to 500 to 600bp, equipped with adapters, treated with bisulfite, PCR amplified, cloned, and sequenced. Comparison of CpG sequences in the tumor and reference genomes could then reveal bisulfite-induced changes in

unmethylated cytosines within CpGs, while methylated cytosines remain unchanged. This method has the advantage that it does not require preselection of regions to be interrogated but the disadvantage of requiring extensive sequencing. The next-generation single-molecule sequencing strategies described previously make this increasingly attractive as a DNA methylation analysis tool.

Chromatin Structure Analysis

Analyses of chromatin structure typically are accomplished by analyzing the DNA sequences that are associated with specific chromatin modification. This can be accomplished using microarray or sequencing strategies.

Chromatin Immunoprecipitation Plus Microarray Analysis

This method, illustrated in Figure 20-12 combines chromatin immunoprecipitation (ChIP) with hybridization to DNA microarrays (chip). ChIP enriches DNA sequences associated with immunoprecipitable chromatin modifications such as histone acetylation (acetyl-H3, acetyl-H4) and histone H3 methylation (dimethyl-H3-K4, dimethyl-H3-K9, and trimethyl-Lys27) for which antibodies are available. The immunoprecipitated DNA sequences are

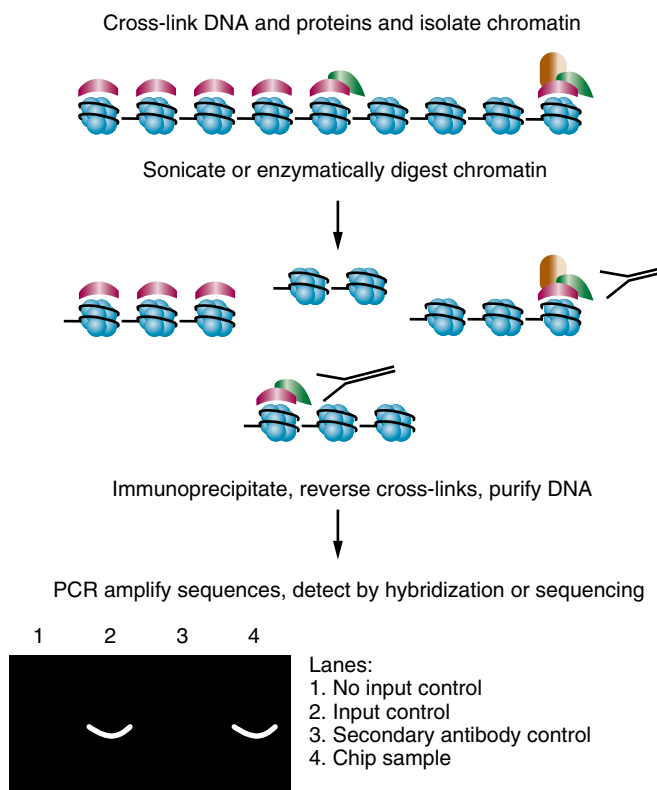


FIGURE 20-12 SCHEMATIC ILLUSTRATION OF CHROMATIN IMMUNOPRECIPITATION ANALYSIS USING MICROARRAYS (CHIP ON CHIP) TO DETECT DNA-PROTEIN INTERACTIONS THAT OCCUR WITHIN INTACT CELLS. The protocol involves formaldehyde-based cross-linking, immunoprecipitation with an antibody to a specific protein, or to an epigenetically modified version of the protein, which precipitates both the protein of interest and the DNA sequence to which it is bound. After reversing protein-DNA cross-links, the presence of specific DNA sequences can be detected by locus-specific polymerase chain reaction with appropriate controls. Alternatively, to detect the loci to which the protein is bound genome-wide, the DNA may be assessed by hybridization to genomic microarrays or may be subjected to massively parallel single-molecule sequencing.

then amplified, labeled, and hybridized to DNA microarrays carrying oligonucleotides distributed along the genome. This approach maps the protein-bound DNA sequences to the genome sequences represented as probes on the microarray.

Chromatin Immunoprecipitation Plus Sequencing (ChIPSeq)

Interrogation of chromatin structure also can be accomplished using a combination of chromatin immunoprecipitation and

high-throughput DNA sequencing (77). In this approach, the genome locations of the DNA fragments recovered by immunoprecipitation with antibodies against chromatin modifications can be mapped using emerging genome wide single-molecule sequencing strategies as described above. This approach has the advantage of being unbiased. However, it requires substantial and redundant sequencing since mapping of chromatin-associated DNA fragments can only be accomplished with statistical accuracy if each fragment is observed many times.

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Transcriptome Analysis

Clinical disease states, including cancer, represent exceedingly complex biologic phenotypes reflecting the interaction of a myriad of genetic and environmental contributions. The characteristics of an individual tumor, reflecting the acquisition of multiple mutations in oncogenes and tumor suppressors in response to various environmental interactions, combined with the inherited germ-line variations that influence tumor growth and response to therapeutic drugs, create an enormous phenotypic complexity. Although the effect of any one of these genetic alterations may be subtle, the combined effect of multiple alterations—together with and in the context of environmental factors, lifestyle, and other factors—can make an important contribution to tumor behavior. It is the aggregate of these effects that leads to immense natural heterogeneity in tumor phenotypes, disease outcomes, and response to therapies. A major challenge is to develop information that can describe this complexity to facilitate an understanding of the disease mechanisms and guide the development and application of therapies. The challenge, as well as the opportunity, of personalized medicine lies in the capacity to develop quantitative data that can match the complexity of the disease.

The analysis of tumor phenotype has traditionally relied on microscopic measures of critical histopathologic characteristics combined with additional biochemical measures, the latter usually involving assays of individual proteins by immunohistochemical methods. These characteristics combine to represent the phenotype of the tumor that can then be linked to clinical outcomes. Unfortunately, in most cases, this phenotypic characterization falls short of matching the complexity of the actual disease process, resulting in broad categorizations that are often imprecise for the individual tumor or patient. Advances in genomic technologies over the past several years have opened the way to addressing the shortcomings of these traditional approaches, providing an opportunity for more precise characterizations of the tumor and the patient.

By far the most powerful of these genome-scale approaches has been the use of DNA microarray analysis to provide measures of the transcriptome of a tumor—that is, the entirety of gene expression information that reflects the activity within a tumor at a given instant in time. Not only do these measures allow for an assay of the activity of essentially all genes within the genome, the much more powerful aspect is the ability to use the information to identify patterns, or profiles, of gene activity that characterize a given phenotype. These patterns have been used to define tumor

subclasses not previously recognized, to predict the aggressiveness of the tumor and disease outcome, and to predict the likely response to various therapeutic interventions (1–6). These analyses can also be used to better understand the underlying biology associated with the specific tumor phenotypes, such as that evident in the expression signatures that reflect the activation of various oncogenic signaling pathways (7). The concept is to take advantage of the complexity of the microarray data to identify patterns that can be associated with distinct phenotypes representing various aspects of the overall cancer phenotype.

The focus of this chapter is to enumerate the strategies that make use of the cancer genome expression data, the opportunities for application, and the benefits already seen in better understanding cancer phenotypes relevant for improved treatment.

Embracing the Complexity of the Cancer Transcriptome

To fully realize the potential of genome-scale information to inform cancer phenotypes requires a shift in the way by which complex, large-scale data is viewed, analyzed, and used. For example, the tradition of identifying one or a small number of biomarkers continues in the context of cancer genomics with the often-held view that expression data is merely a step toward the identification of new biomarkers that can be measured in simple assays. A contrasting view is to consider the expression data in its full complexity as a phenotype itself—a pattern of gene expression (signature) that uniquely identifies a biologic state. The importance of this latter view is the realization that cancer biology and the disease process are complex. Individual risk factors, whether genetic, clinical genomic, or other, represent only single- or low-dimensional snapshots of the disease process and state. What is needed is the integrative view that takes advantage of the full complexity of the data that can be obtained from genome-scale analyses to match the complexity of the biologic phenotypes. Comprehensive, integrative analysis that presents and evaluates these multiple factors and fairly assesses the combined prognostic implications with due regard for the uncertainty that arises when two or more biomarkers conflict is paramount.

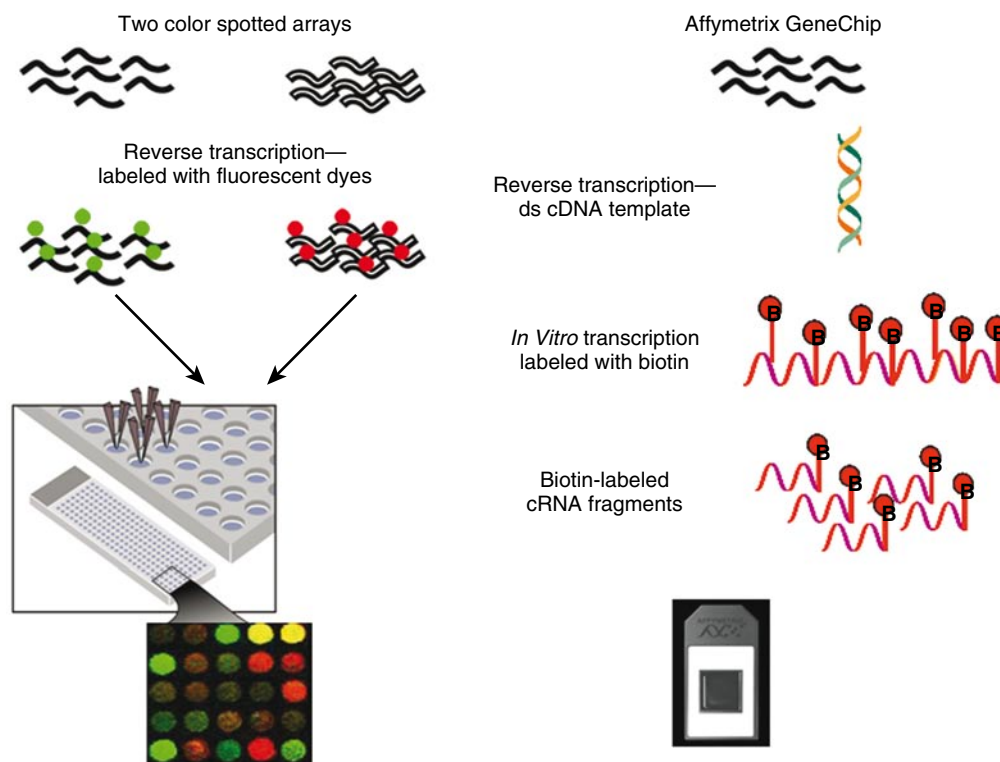


FIGURE 21-1 PRINCIPLES OF ARRAY LABELING AND HYBRIDIZATION. The basic principles of RNA labeling and sample hybridization for the two-color spotted arrays and Affymetrix GeneChip are illustrated.

An example of the importance of adding complexity can be seen in the analysis of survival for patients with non-small cell lung cancer (NSCLC). As with most cancers, the basis for prognosis is clinical staging based on tumor size and the extent of tumor spread. Based on this prognosis, the current standard of care for stage Ia patients is surgery and then observation because clinical trials have not demonstrated a benefit of adjuvant chemotherapy for this group of patients. Nevertheless, approximately 30% of these individuals are in fact at risk for recurrence and death. That the clinical-based classification is indeed imprecise is shown from the results of genomic expression analysis. As depicted in [Figure 21-1](#), a gene expression profile has been developed that can distinguish between high-risk and low-risk NSCLC patients (8). Importantly, a stratification based on this expression profile reveals evidence for extreme heterogeneity within the stage Ia population. As seen in [Figure 21-1](#), individuals classified as stage Ia can exhibit survival characteristics ranging from 90% to 10%. The clinical staging is imprecise and can be considerably improved through the use of gene expression profiles.

Measuring the Transcriptome

The advent of DNA microarray technology allows the study of expression and function of genetic elements at a genome level. When combined with knowledge of the entire repertoire and

components of genes within an organism, this technology has now opened the door to the study of cellular phenotypes in their full complexity. Microarrays also convert the reductionist hypothesis-driven biomedical research to increasingly high-throughput “omics” data acquisition and rigorous quantitative sciences. The application of microarrays in analyzing cancer phenotypes, in particular, has led to an explosion of knowledge on the molecular architecture and heterogeneity of human cancers and provided novel insights into tumor behaviors. Although microarrays are most frequently used to obtain the global gene expression pattern of human cancers, the same technology platforms and conceptual framework are also applied to assay other biologic properties and genetic makeup of human tumors. Here, we aim to summarize the different technological platforms used to analyze gene expression and other biologic information contained in the human cancers and the various approaches to confront and capitalize on this huge amount of information with advanced bioinformatics.

DNA Microarray Platforms

Microarrays can usually be classified into two groups on the basis of fabrication methods and experimental approach. The probes representing gene elements on the microarray can be “spotted” by physical deposition (such as home-made printed arrays or Agilent microarrays) or synthesized *in situ* through “photolithography” (such as GeneChip from Affymetrix). The spotted arrays are frequently used in an experimental design involving a two-color labeling

scheme in which the query sample (for example, a sample of breast cancer) is labeled with one fluorescent dye (such as Cy5) and a reference sample (for example, common reference RNA) is labeled with another fluorescent dye (such as Cy3). The two individually labeled samples are then mixed in an approximate 1:1 ratio and hybridized competitively to the microarrays. Such an experimental design compares the hybridization signals of paired samples with control for various possible variations during the labeling and hybridization procedures and reports expression as the logarithm of the ratio of RNA in a query sample to that in a control sample (Cy5/Cy3). When the paired samples are actual control and experimental samples in biologic experiments, the ratios carry biologic meaning, indicating the alteration of gene expression with experimental perturbations. When a common reference RNA is used in a two-color experiment, the ratios between these two colors contain no intrinsic biologic meaning unless compared with the ratios of the same gene elements with other biologic samples. Before knowledge of the entire complement of genes within an organism was available, amplified cDNA fragments from verified cDNA libraries were often used to represent the gene elements on the microarray. The rationally designed long oligonucleotides are increasingly used to be spotted onto spotted microarrays since they offer better consistency.

GeneChip DNA microarrays from Affymetrix, on the other hand, are usually used with single-color hybridization with each sample individually labeled and incubated onto an array. Many control RNA species with predefined sequences are then “doped” into the samples to control for potential technical variations. The hybridization signals, instead of ratio, generated from any gene elements are reported to represent the expression level of that particular biologic sample. Since the individual probe for genes in GeneChip is short (25 nucleotides), it is possible to distinguish single-base mutation based on their impact on the hybridization signals, thus making it possible to assay for the allelic differences, such as the single-nucleotide polymorphism (SNP).

In addition to obtaining the global gene expression pattern, advances in microarray technology have allowed the understanding of various biologic processes on a global scale. For example, genome tiling arrays have allowed the unbiased and high-resolution definition of transcriptional activities, RNA-binding protein targets, and DNA modifications. It is also possible to profile alternative splicing globally with high-density arrays composed of probes addressing different exons, such as Affymetrix exon arrays. It is also possible to obtain affordable custom arrays based on the individual research needs with the application of maskless photolithography from NimbleGene.

Given the wide variety of array platforms and labeling formats, it is important to determine the reproducibility of measurement of individual genes and the biologic conclusions drawn from different array platforms and different laboratories. Skepticism about the reproducibility of microarray experiments in different laboratories, and comparability of the results on different microarray platforms, have led to concern about the conclusions drawn from the microarray experiments. This issue is especially critical for the use of microarray in the clinical setting for the decision making of patient management. Several studies have now

sought to address the issue of array reproducibility. For instance, a Microarray Quality Control (MAQC) Consortium was formed to experimentally address the key issues surrounding the reliability of DNA microarray data on a large and comprehensive scale. The conclusions from their studies confirm that, with careful experimental design and appropriate data transformation and analysis, microarray data can indeed be reproducible and comparable among different formats and laboratories, regardless of sample labeling format (9). Their data also demonstrate that assays like quantitative reverse-transcription–polymerase chain reaction (PCR) largely confirm the fold change of gene expression observed from microarray experiments. This result validates the utility of microarray as a useful and robust tool for research and, potentially, for the clinical setting.

Microarray Data Analysis

Since each microarray assay typically generates tens of thousands of measurements, extracting biologic information from microarray data demands rigorous quantitation and mathematical modeling provided by various bioinformatics tools. These analytic tools are unsupervised and supervised in nature. The unsupervised analysis methods, such as hierarchical clustering (10) and self-organizing maps (11), are used to arrange genes and samples in groups based on their gene expression in an unbiased fashion, without regard to the nature of the experiment or underlying biology. When unsupervised analysis is used to group biologic samples based on gene expression, it can be used for *class discovery*—the identification of sample classes based purely on the pattern of gene expression. This approach has led to the idea of molecular diagnosis of human cancers based on their gene expression instead of based on the manifested histopathologic features. This analysis allows us to use gene expression patterns to identify tumors with significant clinical heterogeneity indistinguishable from traditional histopathologic features. This approach has led to the discoveries of different subtypes of human cancer not recognized by their histopathologic features. For example, breast tumors can be classified into five major subtypes (luminal A, luminal B, HER2+/ER–, basal-like, normal breast-like) that predict relapse-free and overall patient survival times (12).

The other frequently used analytic approach is supervised analysis in which knowledge about the nature of the samples is used to drive the analysis. A supervised approach for the array data can be used for several purposes. It can be used to identify molecular features associated with tumors with known biologic phenotypes to gain a better understanding of the underlying pathophysiologic mechanisms. This will overcome the confounding technical or irrelevantly biologic variables to identify important association between gene expression and the investigated tumor phenotypes. A gene expression data set might be used to go beyond simple clustering and correlation to derive patterns, or signatures, that represent what we term “subphenotypes,” reflecting phenomena such as hormone receptor status, disease outcome, response to therapies, response to hypoxia, or the activation state of various signaling pathways that contribute to oncogenic progression. It is also possible to prioritize and discover novel biomarkers with

the ability to distinguish tumor subtypes. Finally, a very powerful application of the supervised analysis is to build a predictive model for the purpose of *class prediction* based on the gene expression pattern of the existing samples (training sets). The predictive model can be used to predict their class and likely clinical phenotypes for new unknown samples (validation set; 13). Thus, it is possible to use gene expression to predict the clinical outcomes to guide the diagnostic and therapeutic decisions and realize the possibility of personalized medicine.

The general questions of false discovery and overfitting when analysis draws on expression data of many genes must be faced and addressed in any analysis. Given the extreme complexity of the gene expression datasets, where many thousands of measurements are made on relatively few numbers of samples, structure may be found in the data by chance, and truly relevant structure may be only weakly identified so that resulting prediction in new contexts may be poor. Understanding these issues, and addressing the need to verify results of any exploratory or confirmatory analysis, is critical, and can be addressed from the viewpoints of both biologic interpretation and predictive evaluation in cross-validation and out-of-sample studies.

Functional Annotation of Gene Expression Data

Although the identification of gene expression patterns in tumor samples has greatly expanded the knowledge of tumor biology, it remains a challenge to obtain biologic insights from the long list of genes obtained from supervised or unsupervised analysis. For instance, it is often possible to identify a select number of known genes within a given profile, leading to speculation about the biologic meaning. Nevertheless, this often involves a small number of the total set in the profile raising the concern about the contribution of the other genes—in short, the true context is generally not addressed by these selective analyses. The development of tools that provide a more contextual view of the gene signatures, such as the Gene Set Enrichment Analysis (GSEA) (14), provides an approach to examine whether the genes comprising a gene signature are enriched or depleted for various known biologic activities such

as pathways or other related events. This allows us to examine the likelihood of enrichment of molecular pathways associated with the tumor phenotypes to develop testable biologic hypotheses.

In contrast to the “top-down” approach of direct profiling tumor samples, a “bottom-up” approach using predetermined aggregation of genes (called gene signatures, gene sets, metagenes, or modules) representing known biologic pathways can offer a complementary way to address the characteristics of a tumor phenotypes contained in gene expression on a higher order (Figure 21-2). The gene signatures represent a defined biologic process that can be obtained with defined perturbations or annotated on the basis of existing biologic knowledge. These gene signatures can be used to stratify tumors on the basis of the degree of pathway activities to investigate the relevant biologic phenotypes associated with investigated pathways. This approach has allowed the assessment of activities in oncogenic pathways of human cancers and the grouping of tumors based on these pathway activities and allows the prediction of the effectiveness of pathway-targeted therapeutics based on the degree of pathway deregulation (7). Importantly, these signatures provide tools that can then transfer the in vitro-generated phenotype to an in vivo setting. A cell culture phenotype such as pathway activation is difficult to represent in a diverse sample such as a tumor. In contrast, the expression profiles provide a mechanism to link these two states: The profile represents pathway activation in the cell culture and then can be used to interrogate the expression data from a tumor. In a sense, the gene expression signature becomes the common currency to link the experimental state with the in vivo state.

The importance of this concept is the capacity to generate a series of cancer subphenotypes in the form of genomic signatures that will aid in developing a much more detailed description of the distinction amongst a large number of tumor samples (Figure 21-3). The ability to develop these signatures as relevant subphenotypes will be a key mechanism to understanding the complexities of cancer, including the relevance of the vast array of DNA sequence alterations that will be uncovered in the sequencing of cancer genomes. In a very real sense, this is equivalent to the challenge of linking SNPs with phenotype–genetic association. As in the genetic studies, the more precise the phenotype, the more likely

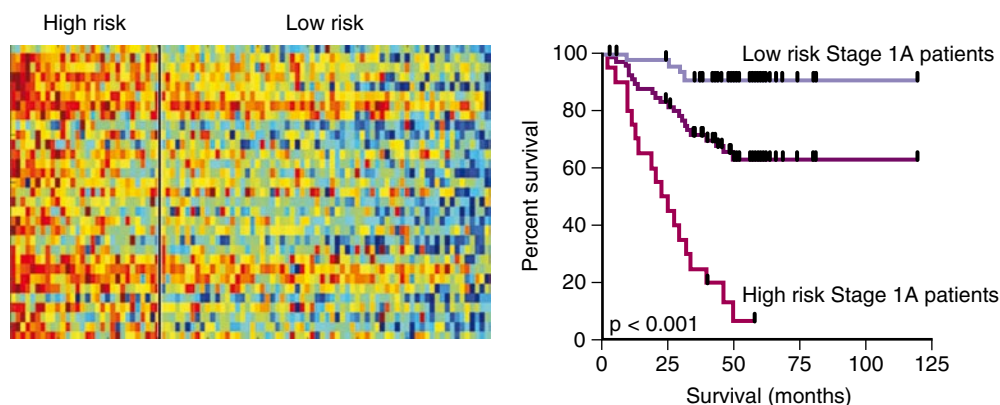


FIGURE 21-2 REFINED PROGNOSIS OF EARLY-STAGE LUNG CANCER USING GENE EXPRESSION PROFILES. The expression profile shown represents one of many selected to predict recurrence in non-small cell lung cancer patients. The right-sided panel depicts a Kaplan-Meier survival curve of stage Ia patients as a group (black curve) and subgroups of this same population of patients identified as at high risk (red curve) or low risk (blue curve) based on the gene expression predictor. Adapted from Potti A, Mukherjee S, Petersen R, et al. A Genomic Strategy to Refine Prognosis in Early-Stage Non-Small-Cell Lung Cancer. *N Engl J Med* 2006;355:570–80.

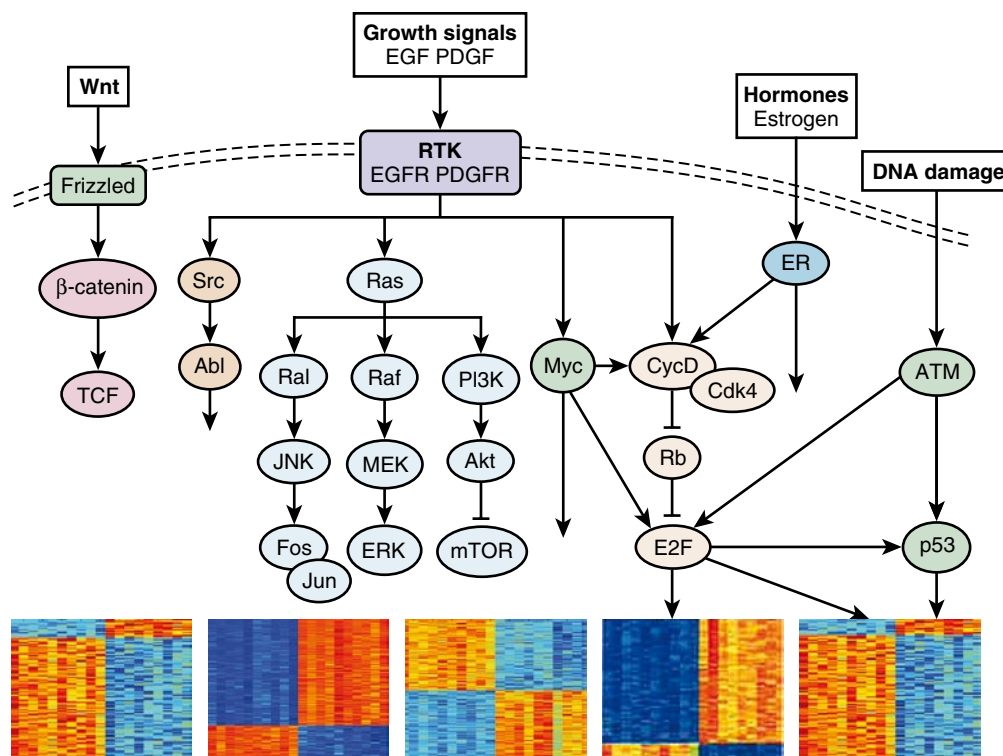


FIGURE 21-3 PATHWAY-SPECIFIC GENE EXPRESSION SIGNATURES. Figure depicts a variety of cell signaling pathways that have been associated with oncogenic processes. Expression profiles are developed reflecting the specific deregulation of individual pathways.

it will be to find such an association. Thus, our ability to generate a series of signatures that can be used as phenotypes will aid the identification of sequence variants that are relevant to these phenotypes (Figure 21-4).

An example of the utility of using gene expression patterns as signatures comes from work at the Broad Institute of Massachusetts Institute of Technology and Harvard where investigators are developing a “connectivity map” based on differential gene expression. The approach is to find nonrandom similarity between genes altered across genetic and/or clinical phenotypes with patterns of gene expression resulting from defined chemical perturbations (15). This approach has already identified associations between hsp90 inhibitors and androgen receptor activity in prostate cancer cells (16) and mammalian target of rapamycin (mTOR) inhibitors and glucocorticoid resistance in leukemia cells (17). Here, shared dependent expression patterns (which can be seen as signatures or expression phenotypes) implicate shared biology and, with the two published applications, facilitate the discovery of novel therapeutic approaches.

Integrative Genomic Analysis

To develop a comprehensive picture of disease states, it is likely that multiple genomic technologies will be brought together in an integrated fashion. Although initial effort on understanding human cancer on a genomic level has focused on the expression of genes encoding proteins, it is becoming obvious that RNA expression alone may not sufficiently assay a disease state to allow accurate disease prediction. There have been many advances in the

development of technology in profiling other genetic properties of human cancers. It is obvious that these data have the potential to further contribute the understanding of tumor heterogeneity. For example, array comparative genomic hybridization (array CGH) has been used to obtain high-resolution measurement and identify regions of conserved gain or loss in cancer samples (18). Several array platforms are frequently used for array CGH. For the spotted arrays, the genetic elements printed on arrays can be the same cDNA arrays used for gene expression studies or the amplification products from defined regions of chromosomal DNA from bacterial artificial chromosome (BAC) libraries. The use of BAC clones makes it possible to directly map the alterations to defined physical locations (18). The array CGH result obtained with cDNA microarray makes it possible to directly integrate and compare with the gene expression results from the same tumors (19). Alternatively, Affymetrix SNP gene chip, originally designed for high-throughput genotyping, can also be used to assess the high-resolution DNA copy alterations in tumors (20). The high-density coverage of the gene chip technology allows the ability to distinguish between the two alleles and make it possible to detect somatic mutations and loss of heterozygosity in cancer tissues.

Array technology has also been used to profile the expression of noncoding (nc) RNAs in human cancers. For example, many studies have focused on the expression of micro-RNAs, a class of ncRNA of 19 to 25 nucleotides in size, which mediates post-transcriptional regulation of their target mRNAs via noncanonical base pairing to play an important in a variety of biologic processes, including differentiation, apoptosis, and oncogenic transformation. Several studies have shown that global

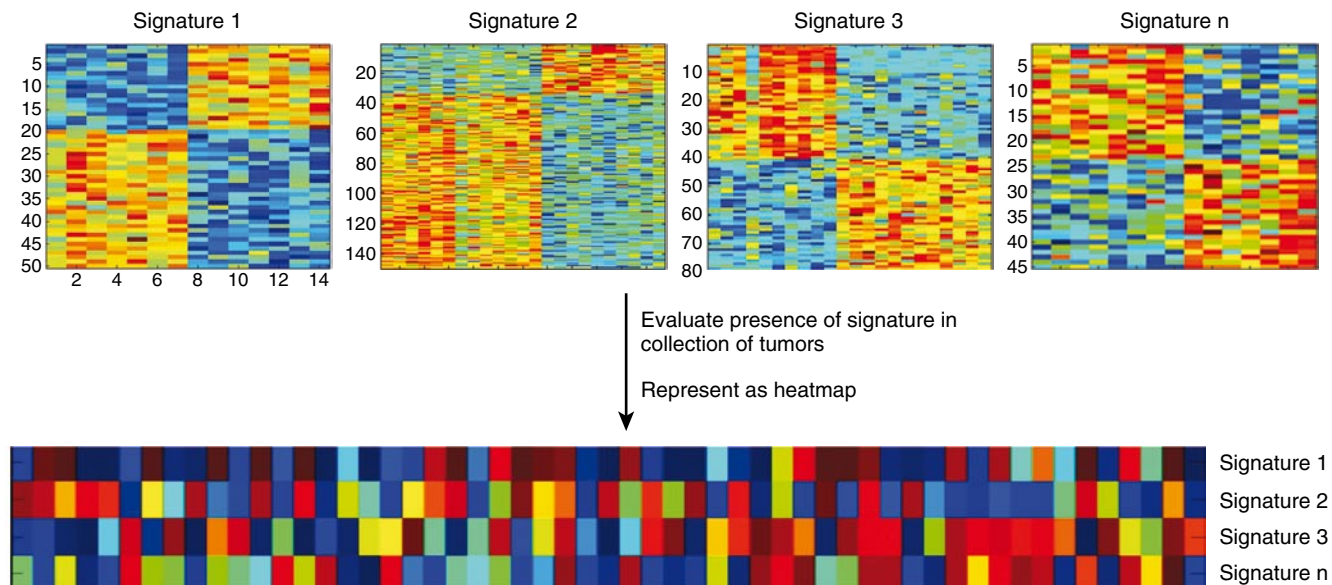


FIGURE 21-4 USING EXPRESSION SIGNATURES AS CANCER PHENOTYPES. The figure depicts a series of gene expression signatures (*signature 1, 2, etc.*) developed using specific biologic perturbations. These can then be assessed in a collection of tumor samples to provide a measure of the phenotype—here expressed as a heat map that reflects probability of the signature within a tumor sample.

expression patterns of micro-RNAs in human cancers can indicate their status and identify subgroups of cancers with distinct clinical outcomes (21). Additionally, other work points to a role for micro-RNA expression in the function of the Myc and Rb-E2F pathways (22,23). These results indicate the role of micro-RNA in modifying tumor behavior and their role in informing clinical outcomes.

The value of obtaining biologic information on a genomic scale from tumors at multiple levels can be illustrated in several ways. First, the integration of tumor gene expression with therapeutic response may allow one to predict the patients likely to benefit from such treatment. For the patients with disease resistant to existing treatment, annotating the molecular pathways from gene expression data may guide the use of pathway-specific adjuvant therapeutic effort for combination therapy. Second, it is possible to obtain additional insights into the molecular mechanisms of gene regulation by integrating different levels of genomic data obtained for the same groups of tumors. For example, by comparing the gene expression studies with array CGH data, researchers have been able to define the relative contribution of DNA copy changes to the dysregulated gene expression and identify gene regulators of gene expression programs and the gene likely to be driving the selective advantage of the amplification (19,20). Third, different biologic information can be combined with the gene expression pattern and clinical information to make the best decision based on the incremental values provided by the additional information. The benefit of integrating diverse information from several different sources has been shown in a research study (24). Importantly, the power of this integrated approach will be further enhanced as more different and diverse information about tumor phenotypes is considered into the decision process. This will lead to a better understanding of tumor heterogeneity and better treatment strategies tailored for individual tumors.

Application of Transcriptome Analysis in Cancer Studies

Microarrays have contributed significantly to the genomics revolution over the past decade and will continue to play an important role in our understanding of cellular biology. Although it is easy to forecast a lasting role for microarrays in clinical and scientific investigation, it remains difficult to anticipate specific applications. Microarrays have been applied to clinical medicine to better understand underlying biology and physiology, to identify marker genes for specific disease behavior, and to improve disease prognosis and treatment. Here we highlight important examples of microarray applications relevant to cancer and how microarrays may improve medical care of cancer patients.

Diagnosis

Expression arrays have been applied to primary human samples of complex phenotypes to identify candidate marker genes for disease, to discover molecular classes of diseases, and to molecularly describe clinical behavior. To identify novel markers for cancer detection or diagnosis, expression analysis was used to detect differences between normal tissues and cancers. Profound differences in gene expression have been found between normal and tumor tissue for most forms of cancer. In a specific example, two groups simultaneously used microarrays to identify a gene overexpressed in prostate tumors compared with normal tumors. The groups validated the gene, called α -methylacyl coenzyme A racemase (AMACR), using immunohistochemistry (25,26). Some clinical pathologists now use AMACR to help diagnose cancer in prostate biopsies, representing a successful evolution from microarray discovery to clinical application.

Expression analysis has also been used to determine if patterns of gene expression correlated with known histologic classification. When this approach was first applied to hematologic malignancies using microarrays (3), strong expression differences between the two major forms of leukemia, acute myeloblastic leukemia (AML) and acute lymphoblastic leukemia (ALL) were found. In ALL, distinct expression patterns associated with the common chromosomal abnormalities underlying leukemia have been identified (27). Similarly, gene expression patterns also easily distinguished follicular lymphomas from diffuse large B-cell lymphomas (DLBCLs) (28) and further classify diffuse B cell lymphomas into two classes based on their origin within lymph nodes; germinal center B-like and activated B-like DLBCL (4). Thus for hematologic malignancies, expression analysis has been shown to accurately reflect cell of origin as well as primary chromosomal abnormalities.

Large differences in global gene expression were also found to exist between solid tumors from different primary sites. Gene expression can reestablish histologic differences between solid tumors from different organs with an accuracy of approximately 80% (29,30). The genes found to best discriminate between tumor types were often tissue-specific rather than tumor-specific, and few genes identified in these screens have become useful clinically. Poorly differentiated tumors that represent diagnostic problems histologically were also difficult to classify using mRNA expression (30). Interestingly, expression patterns of micro-RNA species appear better able to distinguish poorly differentiated tumors from different primary sites (21).

In addition to recapitulating known histologic or genetic traits of disease, microarrays have also identified previously unrecognized molecular subclasses for some solid tumors based purely on unsupervised analysis of their gene expression. Gene expression pattern not only separates the different major types of lung cancer (e.g., small cell v. adenocarcinoma) but can also subclassify the most common form of lung cancer, adenocarcinoma of the lung (31,32). The expression-based classification model that has most dramatically altered the classification of a tumor may be in breast cancers, which can be divided into various subclasses with prognostic and therapeutic implications (12,33). As discussed previously, this work has shown that breast tumors can be classified into five major subtypes (luminal A, luminal B, HER2+/ER-, basal-like, normal breast-like) that predict relapse-free and overall patient survival times (12,33,34).

Whether cancer or other human disease, microarray analysis may redefine diseases from clinical and pathologic collections of findings to molecular entities. Thus, differentiating disease diagnosis on the basis of organ site may start to hold less weight than common underlying biology. The potential for this approach may be realized in anticipating disease outcome and choosing therapy. Diagnosing cancers on the basis of specific pathway activation rather than tissue of origin may allow more effective prognosis and treatment than the diagnostic classifications used currently.

Prognosis

Diagnosis and prognosis often are related in medicine. However, whereas diagnosis focuses on the current disease state, prognosis

focuses on the future behavior of disease in the context of the individual. Investigators have applied microarray analysis to assess whether gene expression patterns correlate with disease outcome and have met with some success.

Oncologists remain optimistic that by understanding the biology and genetics of an individual's tumor, they will be able to accurately predict outcome. Microarrays have identified gene expression patterns that are associated with disease progression and/or patient survival in breast cancer (24,35,36), prostate cancer (37,38), lymphoma (39,40), and lung cancer (8,41). Outcome may be defined as recurrence following definitive surgery, development of metastasis, or death due to disease. Regardless, the preliminary success of the studies mentioned previously suggests that gene expression changes within localized tumors can anticipate recurrence, metastasis, and possibly death due to disease.

Interestingly, although most studies focused on specific types of cancer, expression differences between local and metastatic tumors (of multiple cancer types) were used to also predict outcome (42). In that report, the investigators applied a gene expression signature composed of genes differentially expressed between local and metastatic tumors to successfully predict outcome in breast, prostate, and a type of brain tumor but not in lymphoma, again supporting the idea that some local tumors are preprogrammed for recurrence or progression following local therapy and that microarray analysis can measure this programming and anticipate outcome.

Although initially of scientific interest, microarray-based prognostic models are now being tested for their clinical merit. After two independent groups found that microarray analysis can predict the development of metastasis and survival in women initially diagnosed with localized disease (35,36), clinical trials have started in the United States and Europe to determine how these predictive models derived from microarrays compare with standard risk stratification. Moreover, the 70-gene prognostic signature originally developed by The Netherlands Cancer Center, and now produced by Agendia, has recently gained approval as a diagnostic device by the U.S. Food and Drug Administration (FDA). In addition, outside of clinical trials, microarray-based testing for women diagnosed with localized breast cancer is now available (see <http://www.genomichealth.com>). Furthermore, based on the recent identification of a signature for poor prognosis within early-stage lung cancer (8), a phase 3 trial is in development that will use the genomic prognostic signature to determine therapy.

Treatment

Prognosis identifies individuals at risk and thus in potential need of treatment. Prognosis does not, however, identify the most appropriate therapy for the individual patient. Microarray analysis is starting to be used to direct patient therapy by more accurately classifying the biology and clinical behavior of a patient's disease. Global gene expression can serve as a connective point to associate biologic processes with disease phenotypes and implicate novel therapies. For example, a signature for therapy-induced differentiation in human leukemia cells can be used to identify novel differentiation-causing agents from a screen of 1,739 compounds (43). More recently, it has

been reported that an expression signature of the androgen receptor can implicate hsp90 as a target of therapy (16). Although these efforts remain largely preclinical, they demonstrate how expression can facilitate screens for novel, biologically driven therapies.

Because cancer chemotherapy is toxic and has profound side effects, some of the best examples of targeted treatment using gene expression analysis are found in oncology. Early work found that expression patterns within breast cancer tumors could anticipate sensitivity to a taxane (docetaxel; 44) and anthracyclines (45). Taking a broader approach, it has been shown that chemotherapy response signatures derived from the NCI60 set of cell lines can accurately predict patient response to single agents (docetaxel and topotecan) and combination therapy (6). In the future, perhaps treatment choice will be determined by the molecular biology of the disease as measured by microarrays and other genomic techniques rather than clinical or histopathologic features. It is premature to feel confident that microarray-based tests will be used to determine individualized treatment, but the work performed so far supports this possibility.

Future Directions

As DNA microarray technology and analysis continue to improve, it is important to understand the critical steps that limit our ability to map complex cellular phenotypes using expression analysis and apply microarrays clinically as biomedical assays. DNA microarray

technologies can comprehensively cover the approximately 25,000 genes in the human genome and current technologies likely represent a sufficient sampling of the human transcriptome. Also, there has been robust development of tools for expression analysis that more efficiently and accurately identify informative patterns and/or signatures. The future use of microarrays for disease diagnosis, prognosis, and treatment is more likely to be limited by sampling size, gene annotation, and the onerous work of functional validation than any technical or computational limitations.

In the future, we expect the knowledge and information obtained from the gene expression analysis of human cancers on the bench will make more impact on the management of cancer patients on the bedside. More biomarkers discovered from gene expression analysis will be available to identify the subgroups of cancer patients with different clinical risks to inform therapeutic decisions, improving the treatment response while minimizing unnecessary side effects. But microarrays will make the most impact when the information from the global gene expression of tumors is incorporated into the process of clinical decision making. To formally establish the utility of microarray in the clinical management of cancer patients, it is important to choose the most appropriate decision branch point in the clinical management of cancer patients and test prospectively whether the patients can really benefit from these “genomic” tests. Several studies are under way to test for this possibility. If microarrays do offer the highly expected values and benefits for cancer patients, we believe this will accelerate the realization of the vision of personalized medicine in cancers and other human diseases.

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Mass Spectrometry in Cancer Biology

Proteomic technologies, driven by the human genome sequencing project and the clinical need for molecular understanding of complex diseases such as cancer, have evolved rapidly and accelerated the rate of discovery of molecular processes involved in tumor genesis. The comprehensive study of proteins in disease includes detection, identification, measurement of concentration, characterization of modifications, protein–protein and protein–ligand interaction, regulation, and cellular and tissue-level localization. Over the last decade, studies of these processes have been aided by significant advances in protein/peptide separations and mass spectrometry and genomic/protein databases with supporting bioinformatics techniques and expertise. These technologies allow us to understand cellular processes at the level of the individual protein, multiprotein complex, subcellular compartmentalization, and global proteome level that correlates with and significantly expands information obtained from gene expression studies.

Mass spectrometry is vital to numerous modern proteomics strategies. Although each strategy emphasizes a different aspect of proteomics (e.g., detection, identification, quantification), all are complementary to varying degrees (Table 22-1). One strategy relies on robust peptide separations followed by sensitive tandem mass spectrometry and subsequent matching of peptide fragmentation patterns against predictions from protein sequence databases (“shotgun”). Other strategies are designed to analyze dynamic protein expression in response to perturbation in a system (e.g., the result of experimental treatment or a disease state), and methods such as MALDI (matrix-assisted laser desorption/ionization) imaging mass spectrometry or the use of two-dimensional (2D) gel electrophoresis coupled with mass spectrometry are useful for this analysis on hundreds-to-thousands of proteins, including post-translationally modified or processed forms. In many cases, these approaches can be made quantitative by multiplexing samples that have been differentially labeled using stable isotopes or fluorescent protein tags.

Emerging protein/peptide separation and mass spectrometric technologies have been applied to a wide variety of scientific investigations with emphasis on clinical correlative studies for biomarker discovery, early detection of disease, treatment response, and determination of clinical outcomes (e.g., metastasis, recurrence, and survival). Understanding molecular events that subtend these processes and the discovery and validation of reliable biomarkers will ultimately facilitate novel therapeutic

discoveries and improve patient selection for clinical trials. Achieving these goals, however, will not be easy. Data obtained from proteomic studies are technically and statistically complex, rendering them hard to interpret and difficult to implement in a clinical setting. Nevertheless, one technology that has emerged as vital to the elucidation of protein-driven processes is mass spectrometry (MS). In this chapter, we briefly summarize the current and emerging mass spectrometry-based proteomics technologies, especially with a view to their clinical relevance. We also discuss the challenges faced in the further development of this cutting-edge technology and attempt to provide insight into its future use in clinical studies.

The Role of Mass Spectrometry in Proteomics

It is estimated that the total proteome consists of well over a million different protein species and that the dynamic range of expression of proteins varies over 10^8 to 10^9 orders of magnitude (some estimates in serum are up to 10^{12} ; 1–3). Some proteins (or modified forms) may be expressed during very brief periods during the life of an individual, for example during embryonic development, whereas others may be continually expressed at very low levels (a few copies per cell).

MS has become an indispensable tool for proteomic studies for the detection, identification, and characterization of the protein component of cells, tissues, and organs at any point in health and disease (4–15). For protein analysis, several types of instruments and protocols allow the determination of molecular weight, primary and higher order structure, post-translational modifications, quantification, and localization. Desorption ionization techniques such as MALDI–mass spectrometry (MALDI–MS; 16,17) and electrospray ionization (ESI; 18) have revolutionized our ability to analyze large biomolecules, including peptides and intact proteins, with unsurpassed sensitivity, resolution, and mass accuracy. It is possible to routinely measure molecular weights above 200 kD, obtain accurate, low parts-per-million (ppm) mass measurements on peptides and proteins, fragment individual peptides using tandem mass spectrometry for protein identification, and characterize and map sites of post-translational modifications.

Table 22-1 General Attributes for Common Proteomics Strategies

	Advantages	Challenges
LC/LC/MS/MS (shotgun)	Best sensitivity for protein identification from complex mixtures or proteomes. Low sample consumption (low µg of total protein).	Quantification for large sample sets. Detecting characteristics of intact proteins, including modified/processed forms.
2D gels/MS or DIGE/MS	Quantification on intact proteins including modified/processed forms from large sample sets. Identification of proteins directly from gels.	Requires higher protein amounts (100s of µg of total protein). Most effective for proteins with pI ≈pH 3–11 and MW ≈10–150 kD.
MALDI-IMS or profiling	Proteomic information linked to histology. Relative quantification on intact proteins including modified/processed forms from large sample sets. Fast sample processing with low sample consumption (low µg of total protein).	Identification of proteins is indirect. Most effective with lower MW protein species (≈<40,000).

2D, two dimensional; DIGE, difference gel electrophoresis; IMS, imaging mass spectrometry; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry.

Mass spectrometers measure the mass of biomolecules as charged ions based on their mass-to-charge ratio (m/z). These instruments have three basic components: (1) an ion source that volatilizes and ionizes the analyte, (2) a mass analyzer that separates ions based on their m/z values, and (3) a detector that records the arrival of ions. The most commonly used ionization techniques for biologic applications are MALDI and ESI. Common analyzers coupled with these ionization sources include quadrupole, ion trap, time-of-flight, ion cyclotron resonance, and a combination of these to form tandem (MS/MS) instruments.

MALDI–Mass Spectrometry

Sample preparation for MALDI-MS analysis is simple and quite straightforward. A low volume of peptide or protein sample in solution (<1 µL) is deposited on a target plate, and an equivalent volume of matrix solution (a UV-absorbing low-molecular-weight [LMW] organic compound) is codeposited on to the sample (Figure 22-1A). The molecular ratio of matrix to analyte is typically in the range of 5,000 to 10,000. Upon drying, analyte/matrix cocrystals form (Figure 22-1A), which are subsequently irradiated with UV laser pulses that initiate the desorption and ionization events (Figure 22-1A). One of the key advantages of generating ions by MALDI is that essentially singly charged molecular weight (MW) ion species are formed. The rapid acceptance of MALDI-MS in the field stems from the advantages of simple sample preparation, minimal sample size, ability to analyze a complex mixture such as serum and whole tissues, and high-throughput capability and compatibility with robotics for sample handling and matrix application.

Historically, MALDI has primarily been coupled to time-of-flight (TOF) mass analyzers (Figure 22-1B; 19). A MALDI spectrum typically consists of the average signals coming from several tens to several hundreds of consecutive laser shots (Figure 22-1C). Modern TOF instruments operating under delayed extraction conditions (20,21) are capable of routinely measuring the MW of proteins in the linear mode geometry with high sensitivity (femtomolar) and mass accuracy better than one mass unit per 10,000 for MW below 40,000 (22). Analysis of peptides in the so-called

reflex mode geometry on TOF instruments equipped with an electrostatic reflector (19) routinely offers signal resolutions ($M/\Delta M$) above 15,000 (with ΔM measured at the peak half maximum) and mass accuracy better than 10 ppm for low-femtomolar sample amounts (21,23). Although MALDI-TOF instruments can detect proteins of larger than 250 kD, smaller peptides and proteins in the mass range of 1 to 50 kD are more easily detected. The intensity of the mass signal is semiquantitative depending on the amount of the molecules in the original sample, its ability to co-crystallize with the matrix, and the ionization characteristics of the analytes of interest.

MALDI-TOF instruments equipped with an electrostatic reflector (operating in the reflex mode geometry) have been used to acquire peptide fragment ion spectra by postsource decay (PSD) MALDI (24,25). Although fairly efficient, this technique requires a relatively large amount of starting material to obtain interpretable data. Hybrid QqTOF (26,27) and TOF/TOF (28–30) MALDI mass spectrometers have been designed for fully automated high-throughput protein identification by peptide mapping and fragmentation to retrieve partial or, in some cases, total sequence information. These instruments can operate with MALDI-MS to accurately measure the MW of peptides and with MALDI-MS/MS for fragmenting selected peptides with high sensitivity and low ppm accuracy.

ESI Mass Spectrometry

Ions from peptides and proteins can be formed directly from liquid samples (18). Ions are formed by a spray process by pumping the analyte solution through a thin needle biased at positive voltage toward a grounded sampling skimmer electrode (Figure 22-2A). A continuous spray forms at the tip of the needle and consists of very small droplets that progressively desolve, liberating ions (31). Complete desolvation is aided by passing the spray through a heated capillary (Figure 22-2A) at the entrance to the mass analyzer. For peptide/protein analyses, ESI sources can be operated at flow rates between approximately 0.02 and 1.0 µL/min. Two low-flow rate ESI sources have been described: the micro-ESI (32,33) source, which has an external pump for liquid chromatography coupled with mass

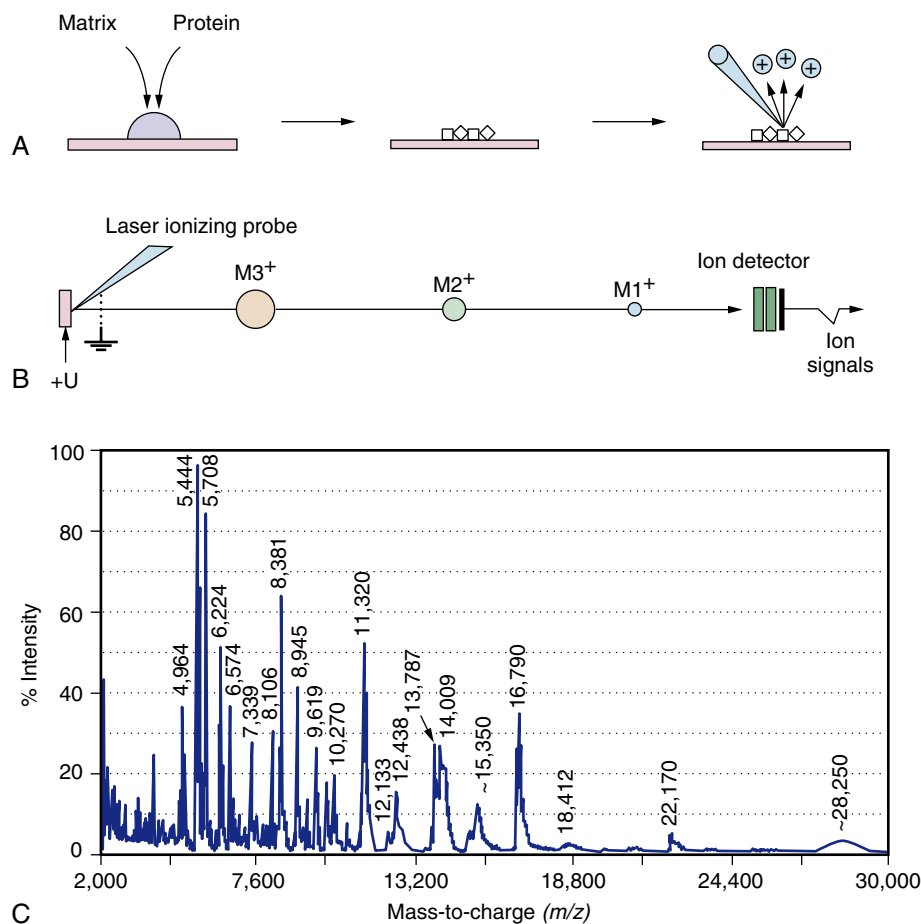


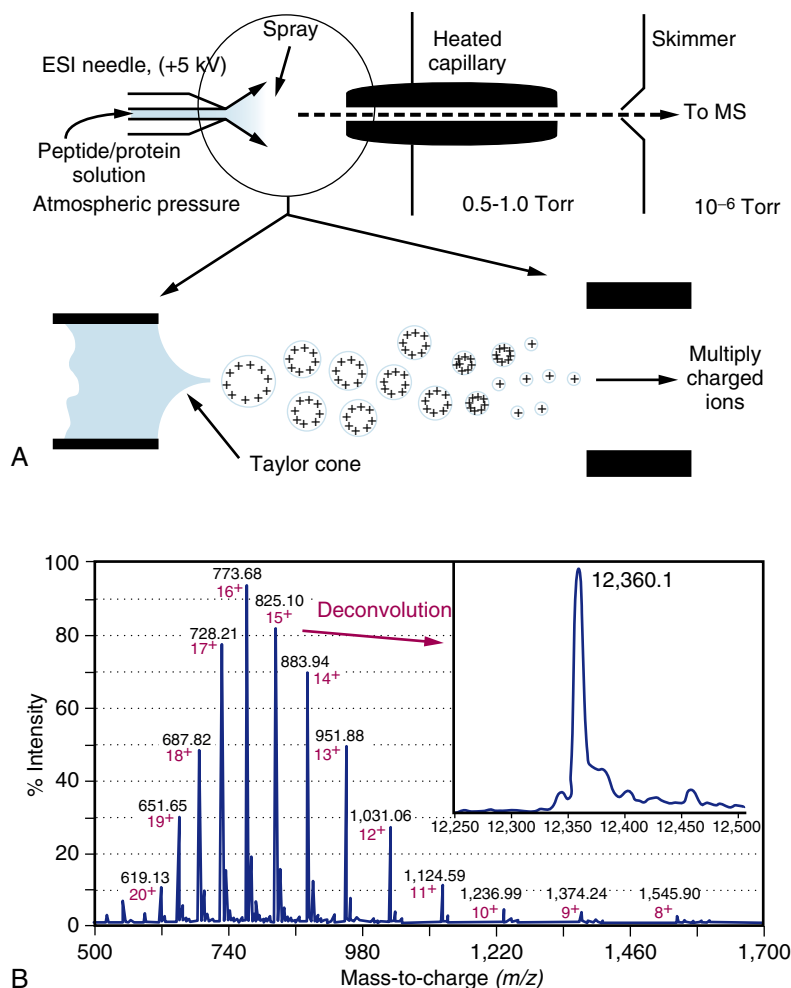
FIGURE 22-1 PRINCIPLE OF MATRIX-ASSISTED LASER DESORPTION/IONIZATION (MALDI) TIME-OF-FLIGHT MASS SPECTROMETRY. **A:** Analyte molecules are first mixed with matrix molecules in a ratio ≈ 1 to 5,000. Upon solvent evaporation, matrix-analyte cocrystals form. In the ion source of the instrument, irradiation of these with a brief laser pulse initiates the desorption-ionization events. **B:** The newly formed ions are accelerated in the source of the instrument by a constant potential difference, giving all of the ions of the same charge the same energy. Ions are therefore separated in time according to their mass as they travel the length of the time of flight. **C:** MALDI time-of-flight mass spectrum obtained in the positive ionization mode from a complex protein mixture. By a large majority, essentially singly charged ions are observed.

spectrometry (LC/MS) or other dynamic flow processes, and nano-ESI (34,35) where atmospheric pressure allows sample flow from an ampoule or other static sample-containing device. The ions are then introduced in the mass analyzer by successive differential pumping stages from atmospheric pressure to high vacuum (Figure 22-2A). Ions so generated can carry multiple charges acquired by proton transfer, having the general formula $[M+nH]^{n+}$. Typically, a single protein will be recorded showing an envelope of peaks of different mass-to-charge ratios measured from the multiply protonated species [i.e., $M+nH/n$, $M+(n+1)H/(n+1)$, $M+(n+2)H/(n+2)$, etc.; Figure 22-2B]. The MW of the sample molecule is then obtained by “deconvoluting” the signal distribution (i.e., for each peak, multiplying by the charge and subtracting the number of protons added; Figure 22-2B). Most often, the m/z distribution for peptide and proteins ions is below m/z 4,000 and centered around m/z 800 to 1,000. Thus for many proteomic applications, mass analyzers coupled to electrospray ion sources need only be able to monitor ions with m/z up to 4,000. One limitation of ESI-MS is that because each sample molecule generates a distribution of ions, it becomes increasingly difficult to analyze and deconvolute overlapping signal distributions from complex mixtures. However, ESI is performed from a liquid sample, and liquid-based chromatographic separation systems such as reverse-phase high-performance liquid chromatography (HPLC) can be directly coupled to mass spectrometers (LC/MS and LC/MS/MS) for mass determination and peptide sequence analyses (36,37).

Commonly, three types of ESI mass analyzers are used for high-throughput proteomic analyses, namely ion traps (38,39), QqTOF (26,27), and Fourier-transformed mass analyzers (FTMSs; 40,41). Ion traps come in various geometries depending on the manufacturer. In all cases these physically “trap” ions in their centers using a combination of collisional cooling with a buffer gas and the appropriate sequential application of potentials on the various electrodes. Once the ions are cooled in the center of the trap, they can be sequentially ejected from the trap according to their m/z ratios and detected. One of the advantages of ion traps is their ability to perform multiple serial tandem mass spectrometry (MS^n) analyses. A parent ion of interest is first selected in the trap and is fragmented into multiple fragment ions. One of these can then be selected and again fragmented into second-generation fragment ions. This cycle can be repeated as long as sufficient ion signal remains. In the case of peptides, this approach may allow gathering of better sequence information or precisely localizing post-translational modifications (42–46). In modern ion trap instruments such as high-capacity or linear traps, the sequence of events from MS to MS/MS measurements is very fast (≈ 0.1 – 0.2 seconds). These instruments are ideal for high-throughput proteomic analyses in the LC-MS configuration (39,47). Although ion traps offer very high throughput and sensitivity, they lack mass resolution and accuracy (typically above 100 ppm). In addition, in the MS/MS mode, these are limited to measuring m/z ratios down to only about one third of the parent ion mass, thereby missing low m/z fragment ions and immonium ions that can yield important sequence

FIGURE 22-2 PRINCIPLE OF ELECTROSPRAY IONIZATION (ESI) MASS SPECTROMETRY.

A: Analytes in solution are pushed through a needle biased at a few kilovolts. This needle faces a grounded capillary. An electrospray forms due to the potential difference applied between the tip of the needle and the capillary as the analyte solution exits the needle. The droplets formed in the spray progressively dissolve, ultimately liberating (multiply) charged ions. These enter the mass spectrometer by a series of differentially pumped stages. **B:** ESI mass spectrum of cytochrome-c acquired with an ion trap instrument showing the multiply charged signal distribution and the deconvoluted spectrum.



information in some applications. FTMS instruments currently provide the highest mass measurement accuracy available for structural characterization of peptides, proteins, and other biomolecules (40,48,49). One of the advantages of FTMS is the very high resolving power, typically between 200,000 and 1,000,000 depending on the strength of the magnetic field, providing mass accuracy routinely better than 1 ppm for lower MW analytes. FTMS instruments can also be used to effectively sequence peptides by collision-activated or- induced dissociation (50) or infrared multiphoton dissociation (IRMPD), and electron capture dissociation (ECD; 41,51–53). As with ion traps (54) and QqTOF (55,56), LC-MS (and LC-MS/MS) can be performed using FTMS but the duty cycle limits the abilities of the instrument to effectively perform a fast scan of the LC run and subsequently perform MS/MS measurements. To partially circumvent this limitation, hybrid ion trap/FTMS instruments have been developed. In this case, peptides from the LC run are selected and fragmented and the resulting ions analyzed in the ion trap while the ICR cell is used to accurately measure the MW of the parent ions (thereby achieving higher cycle rates; 57). Also the accurate MW determination of peptides correlated with their LC retention time and fragmentation pattern has been found to be sufficient information in many cases to unambiguously identify the

corresponding protein (58,59). The newly developed ion trap/orbitrap mass spectrometers (60) are capable of performing similar measurements (61–63).

Proteomic Applications and Strategies

Mass spectrometry is often used to perform three general types of measurements: protein profiling, protein identification, and protein quantification.

Protein Profiling

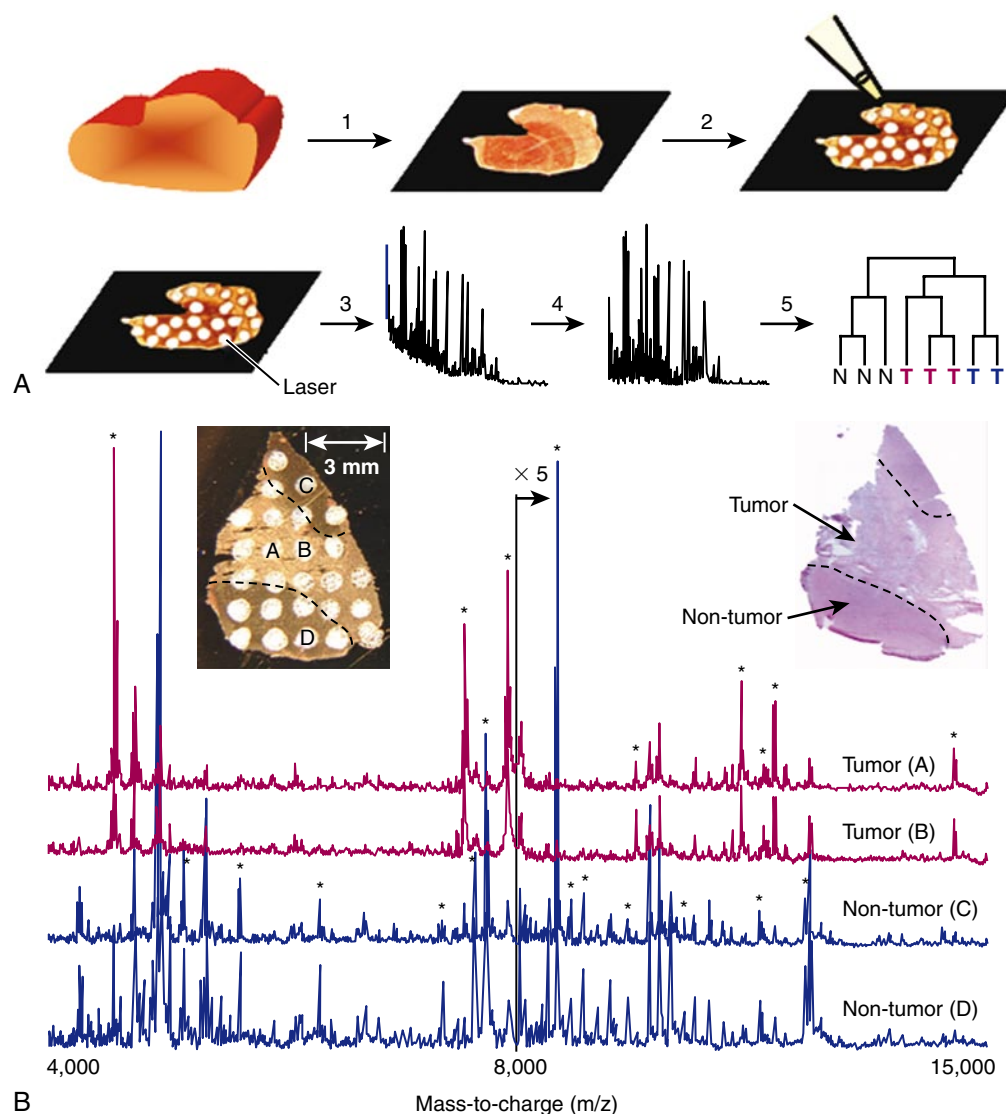
Two platforms may be used for profiling experiments. In some cases, MALDI-TOF MS is used to monitor complex mixtures because of its speed and ease of use. In other cases where sample complexity requires a prefractionation step, multidimensional LC-MS is used. Protein profiling (i.e., the recording of the protein pattern of a sample) can be performed on complex peptide or protein mixtures from whole- or partially fractionated cell and tissue extracts (64,65); biofluids such as serum (66), plasma (67–69), urine (70,71), cerebrospinal fluid (3,72), and

directly from thin tissue sections (11,73). Mass spectra of proteins within these fractions represent a “profile” of the protein population of the sample. Each signal within these profiles corresponds to a unique protein species and its intensity under normal conditions is generally proportional with its abundance. Comparative studies of profiles of samples from control and disease can provide statistically significant patterns unique to each (74–77). Such profiles, however, only yield MW information for individual peaks in the spectrum. In most instances, MW information alone is not sufficient for unambiguous protein identification. The profiling strategy, however, allows for the direct discovery of protein features in the spectra that may correlate with diagnosis, progression, therapy, and outcome.

One of the MALDI-based profiling platforms used for serum and other biofluid analysis is termed “surface-enhanced laser desorption/ionization (SELDI) mass spectrometry” (78–80). SELDI uses the MALDI process with a commercial sample preparation step. Partially purified peptides and proteins from a complex protein mixture are selectively bound to a coated surface based on properties such as hydrophobicity, anionic or cationic charge,

and metal affinity (81). After the addition of matrix and subsequent desorption/ionization by the laser, the ions are analyzed by TOF-MS. Although the sample binding step to activated surfaces simplifies the preparation procedure, it often lacks reproducibility, generating inconsistencies in comparative measurements due to variations in sample constituents and affinity surfaces (82,83). Moreover, loss of low-abundance proteins during sample preparation has been observed (84). Nonetheless, this platform has been used for protein profiling studies by a number of laboratories.

Direct analysis of proteins in tissues using MALDI-MS can be performed on thin sections cut from fresh-frozen biopsies (Figure 22-3A; 73,85–87). Sections are cut in a cryostat and thaw-mounted on a target plate. The energy-absorbing matrix is then manually or automatically deposited on the section and after irradiation by the laser, 300 to 1,000 individual protein signals can be observed (88). Protein profiles obtained from sections can be compared, and statistically-significant signatures indicative of disease can be identified (74,77). Figure 22-3B presents four protein profiles obtained from a 12- μm -thick human grade IV glioma biopsy section analyzed in this way. A serial section from the same biopsy



was stained with hematoxylin and eosin (H&E) (Figure 22-3B) for correlation with visible cellular morphology. The protein profiles obtained from the tumor (spot A and B) and tumor-free areas (spot C and D) show significant differences. The next step involves identification of the spectral features in this signature to elucidate the individual protein “markers.”

On the basis of the direct tissue analysis by MALDI-MS, several studies have been published from a variety of tumor tissues (11,74,77,89). Data were collected using standard instrumental acquisition and processing parameters including calibration, baseline correction, and smoothing. A more detailed description of the statistical analysis process has been described elsewhere (74,77). Mass spectra were analyzed using unsupervised and supervised hierarchical multivariate cluster analyses to subclassify the samples according to their expression patterns and to look for relationships between tumor subtypes and clinical outcome. Class prediction models were then created using the protein profiles from a training cohort of tumor and control samples, and finally a test cohort was analyzed using another set of samples.

Figure 22-4A shows an example of tumor classification based on statistically-significant differences observed in protein profiles. In this example, through the expression of 41 protein signals among the several hundreds observed, it is possible to discriminate with a classification accuracy of 99% grade IV gliomas (49 biopsies) versus nonmalignant brain biopsies (19 biopsies) with a classification accuracy of 99% (77). In this same study, using a data set of 108 glioma patients, two patient populations, a short-term and a long-term survival group, were identified on the basis of tissue protein profiles (Figure 22-4B). A summary survival score was determined for each patient with subsequent assessment of short-term and long-term prognoses. A protein pattern of 24 distinct signals distinguished patients on the basis of survival trends from the time of diagnosis into two groups, short-term survival (mean survival, <15 months) and long-term survival (mean survival, >90 months). In addition, a subset of 57 patients with high-grade, grade IV, malignant gliomas was studied to determine whether protein patterns could further differentiate patients based on survival from the time of glioblastoma multiforme presentation (inset, Figure 22-4B). A distinct pattern was identified that segregated these patients into a shorter-term survival group (average survival, 10.9 months) and a longer-term survival group (average survival, 16.8 months). This is particularly interesting given that these patients were indistinguishable on the basis of histology.

Tissue sections can also be systematically investigated in a “global” analysis procedure by MALDI-imaging mass spectrometry (IMS; 90–92). In this case, matrix is deposited in an X/Y Cartesian array over the entire section, and MALDI measurements are performed in the matrix array at a fixed resolution over the entire surface of the section. Ion-density maps can then be created for every signal detected by plotting each protein-signal intensity as a function of tissue coordinates. From a single scan across a section, hundreds of density maps, or images, can be obtained, each corresponding to a different protein species present in the specimen.

A protocol to selectively profile proteins from cancer cells present in fine-needle aspirates by MALDI-TOF MS has been reported (93). The advantage of this approach is that protein

information can be obtained from tissue abnormalities observed from computed tomography (CT) scans, magnetic resonance imaging (MRI), or radiographic images with a minimally invasive, well-established procedure. Collected clumps of cancer cells can then be directly analyzed by MALDI-MS and the resulting profiles statistically interrogated. This procedure results in the production of high-quality, cancer-cell-specific protein profiles and can be applied to other types of disease conditions. Ultimately this approach has the potential to be useful for clinical diagnosis, classification, and individualized treatment of patients.

Protein Identification

Identification of proteins from complex mixtures by mass spectrometry can be accomplished by strategies that are often referred to as “top-down” and “bottom-up” approaches (Table 22-1).

Top-Down Proteomics

Top-down proteomics generally begins with the purification of a given protein using separation methods such as reverse-phase-high-performance liquid chromatography (RP-HPLC) and 1D and 2D gel electrophoresis. The isolated protein is then submitted to site-specific enzymatic digestion (e.g., trypsin) to generate discrete peptides. Peptide mass mapping using MALDI-TOF MS in high-resolution mode may be used to produce mass spectra having high mass accuracy (<10 ppm). The pattern of peptide-ion masses generated by protease digestion is termed a “peptide mass map” and is used to query protein databases for protein identification (94–98). Identification of a protein on the basis of peptide maps can be further validated by sequence analysis of some of the higher abundance peptides using MALDI-PSD, MALDI-MS/MS, or ESI MS/MS. Sequencing of some of the unassigned peptides may also be useful to discover or confirm the presence of post-translational modifications such as acetylations and phosphorylations as well as characterizing possible sequence variations (99–102).

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE, 2D gel) has been the protein-separation technique most associated with top-down proteomics, whereby several thousand proteins in a single sample are first resolved by charge (isoelectric point, or pI) using isoelectric focusing (IEF), followed by an orthogonal second-dimension resolution based on apparent molecular mass using sodium dodecyl sulfate (SDS)–PAGE (Figure 22-5). The excellent resolving power of this technique has facilitated the identification of the major proteins in a tissue or subcellular fraction by MS methods. In addition, 2D PAGE has been used to compare relative abundances of proteins in related samples, such as control and diseased, allowing the response of classes of proteins to be determined. This differential-display experiment is best accomplished using multiplexing and internal standards, where protein changes can be quantified without interference from technical (gel-to-gel) variation, and from a variety of conditions each measured in repetition for statistical confidence (see DIGE in subsequent sections). Resolved proteins can then be directly subjected to

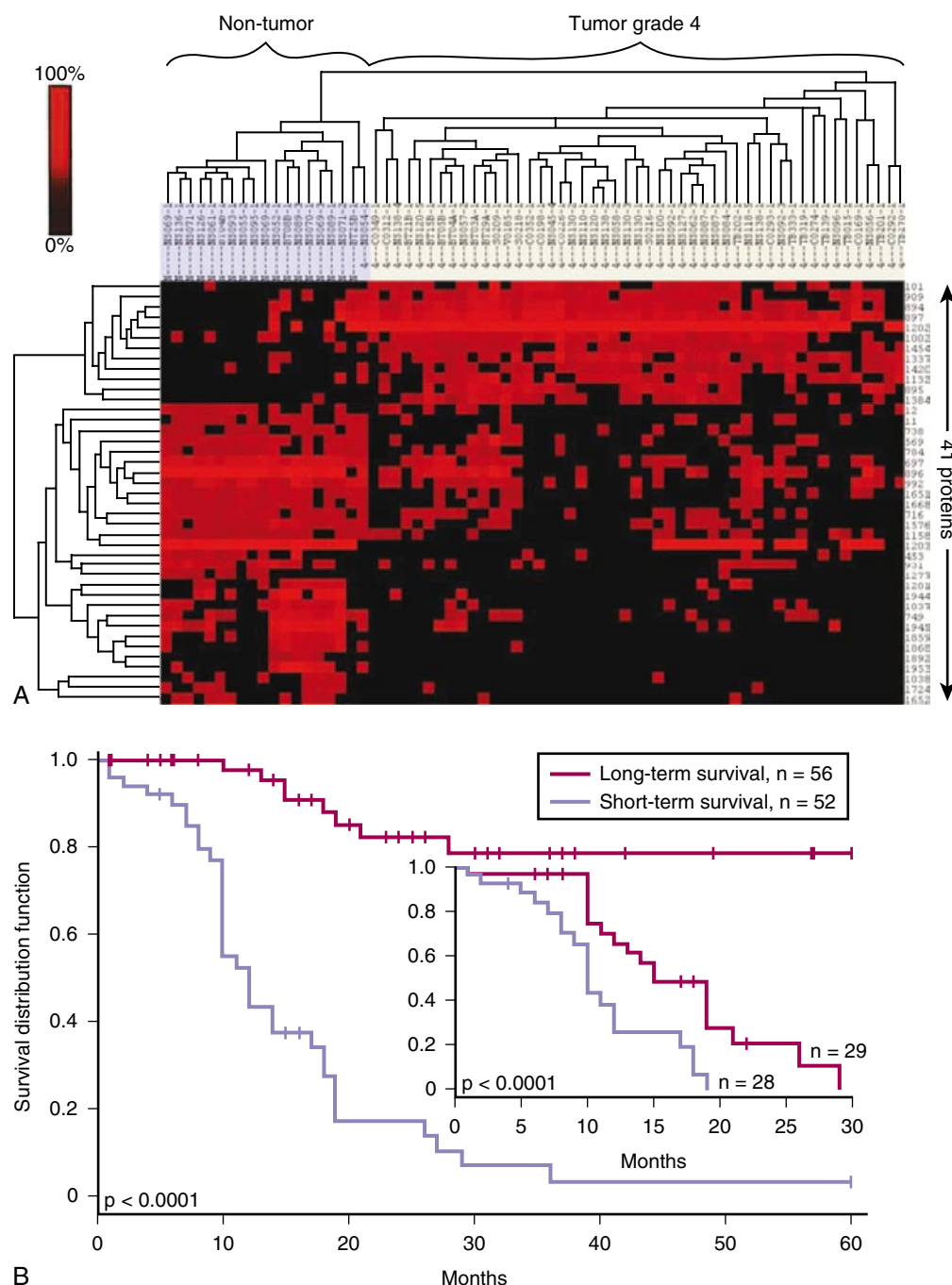


FIGURE 22-4 A: Hierarchical cluster analysis of normal brain and grade IV gliomas. Tissues were clustered according to the protein expression patterns determined by statistical analyses. Clustering was based on 41 differentially expressed protein signals. Each row represents an individual protein signal and each column represents an individual patient tissue sample. The dendrogram (top) clusters tissue samples on the basis of similarity in protein expression profiles. The intensity of each mass signal is indicated by a black-to-red scale, with black denoting the lowest intensity. **B:** Kaplan-Meier survival curves for patient groups with a short-term or long-term prognosis according to mass spectrometry (MS) proteomic patterns. Analyses were done to determine discriminatory protein patterns that stratified, on the basis of patient survival trends, all patients with gliomas from the time of initial pathologic diagnosis using 24-protein signals and patients with grade IV glioblastoma from the time of glioblastoma multiform presentation using two-protein signals (inset). $p < 0.0001$ for each analysis.

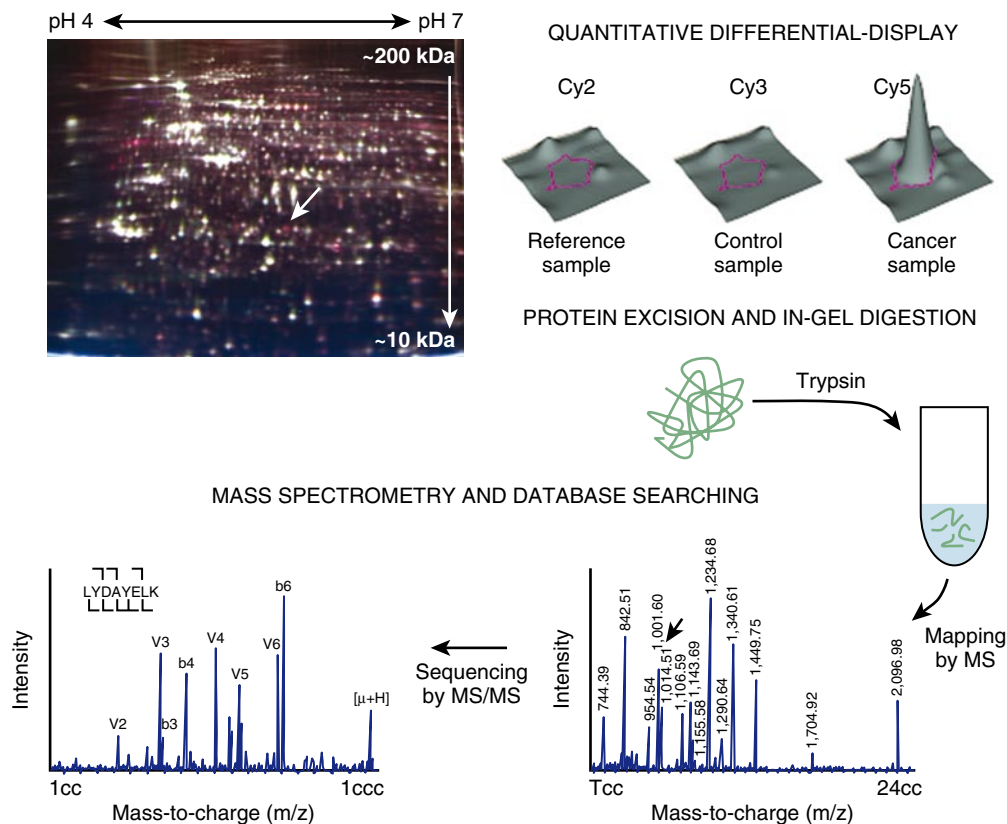
identification strategies as described by digesting the proteins in gel and extracting the resulting peptides for mass spectrometry (provided that the stains used for protein visualization are compatible with these subsequent steps).

Bottom-Up Proteomics

Bottom-up proteomics, also termed “shotgun proteomics,” is accomplished by first digesting a sample (which could contain a simple or complex mixture of proteins) with a protease and subsequent separation of all of the resulting peptides by liquid chromatography.

Multidimensional liquid chromatography consists of prefractionating peptides first according to their net charge using strong cation exchange chromatography (typically seven to ten fractions) and second, according to their hydrophobicity by RP-HPLC coupled online with an ESI mass spectrometer (Figure 22-6A). Peptides can be mass analyzed and fragmented “on the fly” as they elute from the LC column (Figure 22-6A). A peptide mass analysis is first performed followed by one or several MS/MS scans whereby peptide ions undergo collision with an inert gas, and the resulting peptide ion fragments are recorded. Thus, each selected peptide generates

FIGURE 22-5 WORKFLOW FOR A DIFFERENCE GEL ELECTROPHORESIS–MASS SPECTROMETRY (DIGE/MS) ANALYSIS. Clockwise from upper left: A false-color DIGE gel containing three prelabeled protein samples is shown. In practice, the mutually exclusive CyDye channels are imaged separately and used to generate spot-volume density maps at 100- μ m resolution (example shown for Cy2-reference, Cy3-control, and Cy5-cancer samples from the resolved protein indicated by white arrow in the gel). The intragel Cy2: Cy3 and Cy2: Cy5 ratios are then compared across multiple DIGE gels (each containing two Cy3- or Cy5-labeled samples and a Cy2-labeled reference aliquot), and the ratios are normalized using the Cy2 value for each resolved protein, enabling statistical analysis (student *t* test, analysis of variance [ANOVA]). Proteins of interest are then excised and digested within the gel-plug with trypsin protease. The resulting peptides are subjected to matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS; “mapping”) and data-dependent MALDI-MS/MS (“sequencing”), and the mass spectral data are used to search protein databases to identify significant candidate protein matches.



a fragment ion spectrum that serves as a “fingerprint” for identification. This cycle is repeated until all of the peptides have eluted from the chromatography column. The fragmentation spectra are then compared with *in silico*–generated MS/MS spectra derived from peptides of the same nominal MW encoded in a protein database (Figure 22-6B; 54). Computer algorithms are commonly used for this data-intensive process, with some of the more commonly used commercial solutions, including Sequest (Thermo Scientific; 103,104) and Mascot (Matrix Science; 105), and open-source solutions such as X! Tandem (Global Proteome Machine Organization; 104,106). The identification of two or more peptides from the same protein is often considered to be the minimal requirement for a reliable identification of its presence in the original protein extract (107). Although LC-MS/MS analysis is robust and reliable, it is also time-consuming due to the required multidimensional LC separations. Optimal LC-MS/MS results are obtained when analyzing samples of limited complexity, such as bands cut out from a gel or protein complexes obtained by immunoprecipitation. Technology improvements such as high-cycle-duty ion-trap systems have greatly improved the time required for analysis (39,47). Thus, at a given time point during the LC run, one MS scan followed by multiple MS/MS scans of peptides with different *m/z* values can be done in less than one fourth of a second, allowing the analysis of more complex protein mixtures (47).

Protein Quantification

Several mass spectrometry–based approaches have been developed that allow for the relative or absolute quantification of

proteins (Table 22-2). Most protocols involve the use of stable isotopes to differentially label proteins or peptides prior to mixing samples for multiplexing onto the same analytical run (108–115). This negates instrumental variations and enables direct quantification of the same *m/z* values between the different isotopic labelings (ionization efficiently is not affected by isotopic composition). These *in vitro*–labeling strategies are, however, susceptible to technical variation introduced during the protein/peptide labeling and enrichment steps and therefore require replicate analyses. One well-established technique is referred to as isotope-coded affinity tagging (ICAT), which uses stable heavy and light isotope affinity tags that are reactive toward cysteine residues (108). The tagging is performed on the intact protein prior to enzymatic digestion whereby the sample and reference protein extracts are tagged with either the light or the heavy tag, respectively. The extracts are then mixed and digested with a protease. The ICAT tags typically contain a biotin group that allows the separation of the tagged peptides using a streptavidin affinity purification step. The tagged peptides are then analyzed by LC-MS and quantification performed by mass spectrometry by monitoring the intensity of peptide pairs that are separated by the mass difference expected between the light and heavy tags. The identity of the corresponding proteins can be obtained by fragmenting the peptides by MS/MS and performing database searches as described previously. *In vivo*–labeling methods are also available that involve growth in a defined medium containing a heavy isotope (116) or labeled amino acid (117) to

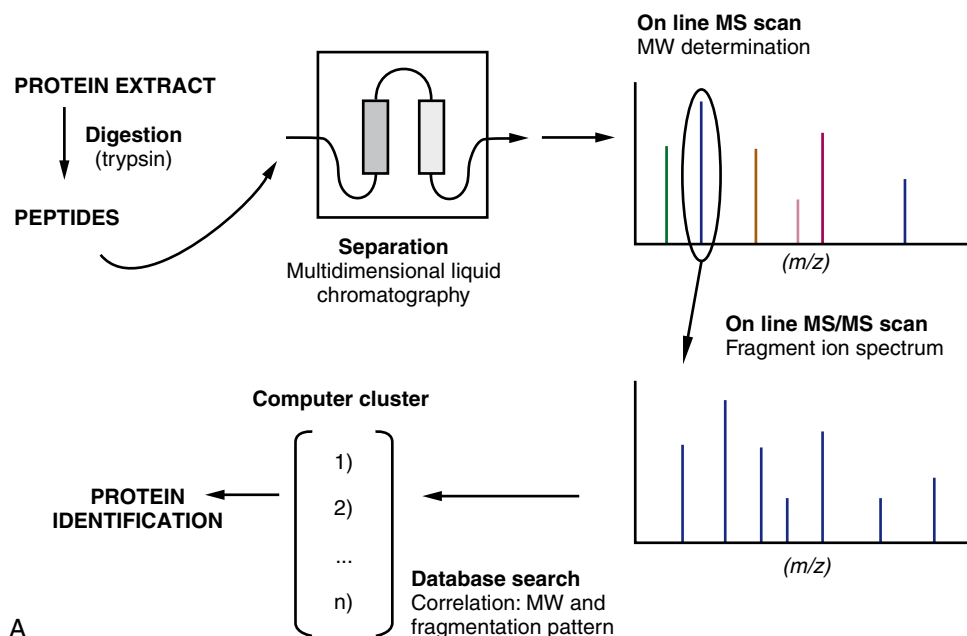
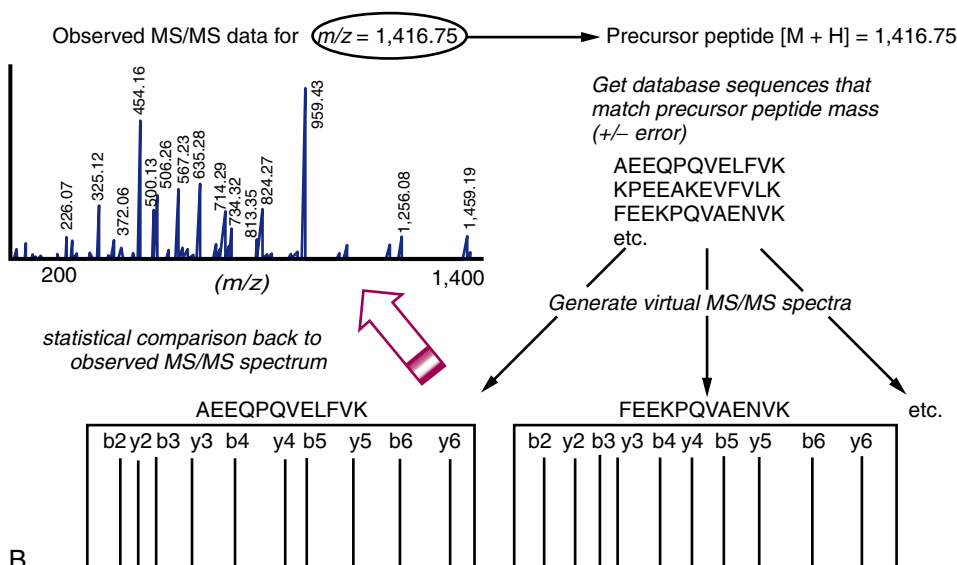


FIGURE 22-6 BOTTOM UP PROTEIN IDENTIFICATION BY LIQUID CHROMATOGRAPHY (LC)-MASS SPECTROMETRY (MS)/MS FROM A COMPLEX PROTEIN MIXTURE. A: The sample is first digested (by trypsin) and the resulting peptides are separated by multidimensional liquid chromatography (typically strong cation exchange followed by reverse-phase separation) coupled online to the mass spectrometer. As they elute, the mass-to-charge ratios of the peptides are first determined followed by one or several MS/MS scans from the most abundant peptide signals. This cycle is repeated until all of the peptides have eluted from the chromatography column. **B:** Example of an MS/MS database search. For each selected precursor peptide (example shown for m/z 1416.75), peptides of similar nominal mass are extracted from sequence databases and predicted fragmentation patterns are derived in silico. These patterns are then compared with the observed fragmentation spectrum to generate correlation scores.



achieve stoichiometric labeling. Although these are not suitable for human biopsies or other tissue samples, they introduce the lowest amount of technical variation to an experiment owing to the fact that samples are multiplexed prior to protein extraction and subsequent manipulation. Recent “label-free” variations for quantitative LC-MS/MS strategies rely on peak intensity measurements of peptides detected by mass spectrometry (118–120) or on the number of ions per protein detected in a mass spectrometric experiment (121).

Strategies based on 2D gel technologies for differential-display proteomics have been successful for analyses on a global scale. Multiplexing samples labeled with stable isotopes have been used in gel-based proteomics (122), and similar to the shotgun strategies

described previously, quantification is performed at the level of the peptide digests during mass spectrometry, where MS data are acquired for each resolved protein.

Difference gel electrophoresis (DIGE) technology adds an essential quantitative component to 2D gel-based proteomics to a level whereby even subtle changes in protein abundance and charge-altering post-translational modification (such as acetylation and phosphorylation, among others) can be monitored from multiple experimental conditions with statistical confidence (Figure 22-5; 123–127). DIGE overcomes many of the limitations commonly associated with 2D gels such as analytical (gel-to-gel) variation and limited dynamic range that can severely hamper a quantitative differential-display study. This is accomplished by

Table 22-2 Mass Spectrometry Technologies for Relative and Absolute Quantification of Proteins

Technique	Site Labeled	Quantification	Linear Dynamic Range	Sensitivity	Reference
ICAT	Cysteine Sulfhydryls	Relative	10 ³	Low fmol	108
iTRAQ	Free amines	Relative	10 ³	Low fmol	114
AQUA		Abs	10 ³	Low fmol	146,115
SILAC	In vivo labeling	Relative	10 ³	Low fmol	117,147
Label-free spectral counting	None	Relative	10 ³	Low fmol	118,119,120,121,148
MALDI IMS		Relative	10 ²	High fmol to low pmol	90,91
DIGE	ε-amines, Cysteines Sulfhydryls	Relative	10 ⁴	Mid to low fmol	126,125
Metabolic labeling	In vivo labeling	Relative	10 ³	Low fmol	116,117

AQUA, absolute quantification (of proteins); DIGE, difference gel electrophoresis; ICAT, isotope-coded affinity tagging; IMS, imaging mass spectrometry; iTRAQ, isotope tags for relative and absolute quantification; MALDI, matrix-assisted laser desorption/ionization; SILAC, stable isotope labeling with amino acids in cell culture.

multiplexing samples that have been prelabeled with spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) into the same analytical run (2D gel), as this removes gel-to-gel variation from the quantitative measurements made for each resolved protein between the three dye excitation/emission spectral channels. Only those proteins displaying statistically significant alterations need be excised and subjected to the MS-based protein identification strategies described previously.

However, single-gel multiplexed experiments have limited statistical power, and DIGE is most advantageous when one of the dyes is used to label a pooled-mixture containing an equal aliquot of all samples in a multigel experiment that co-analyzes independent replicate samples from multiple conditions. The pooled sample is labeled with Cy2 and partitioned out to each gel to provide a unique internal standard for every protein resolved in the gel; the Cy3 and Cy5 dyes are then used to label individual samples for multiplexing two samples onto the same gel along with the internal standard gels (128,129). Although direct quantification is performed between the Cy dye channels within a gel without interference from gel-to-gel variation, this is not performed between the two individual samples coresolved in the same gel. Rather, the Cy3: Cy2 and Cy5: Cy2 ratios for each protein are normalized across all of the gels in a large experiment, using the Cy2 signals for separate normalization of each protein under survey. This also allows replicate samples from multiple conditions to be intercompared using univariate statistical analyses (student *t* test; Figure 22-7B, analysis of variance [ANOVA]) as well as multivariable statistical analyses (principle component analysis, hierarchal cluster analysis; 130,131).

The feasibility and benefit of the DIGE/MS approach have been demonstrated in a number of preliminary studies using small patient cohorts in colon (129), liver (132–134), breast (135,136), esophageal (137,138), and pancreatic cancers (139) and has begun

to be applied to larger patient cohorts (140). Many studies use laser capture microdissection (LCM) to procure a highly enriched population of the cells for analysis (141–144). Multivariate statistical tests (principle component analysis, unsupervised hierarchical clustering, *k*-means) can now be applied to DIGE datasets to identify subclasses of proteins that can discriminate between biologic conditions, assess the biologic relevance of a list of proteins, identify sample outliers, and elucidate prognostic and diagnostic disease markers (130,131,140).

Complementary Technologies

No single proteomics technology platform is capable of accomplishing a global analysis of the entire proteome, but several platforms often provide complementary information. For example, direct tissue profiling requires only a small amount of material to produce semiquantitative information on hundreds of intact proteins and modified forms directly from the intact tissues. When coupled with unsupervised multivariate statistical clustering algorithms, the patterns produced can provide robust prognostic and diagnostic disease markers (74,77) even though a fraction of the proteome is covered.

MALDI-IMS and 2D-DIGE/MS may be used for quantification of intact proteins, visualizing biologically significant alterations in abundance, processing, and post-translational modification. As DIGE is built upon 2D gel technology, proteins of interest can be readily identified using standard top-down MS-based approaches directly from the DIGE gels, and medium-range (e.g., pH 4–7) and narrow-range (e.g., pH 5–6) IEF focusing with a commensurate increase in the amount of protein can be used to increase the depth of analysis. However, these analyses typically require hundreds of micrograms of material per sample for a

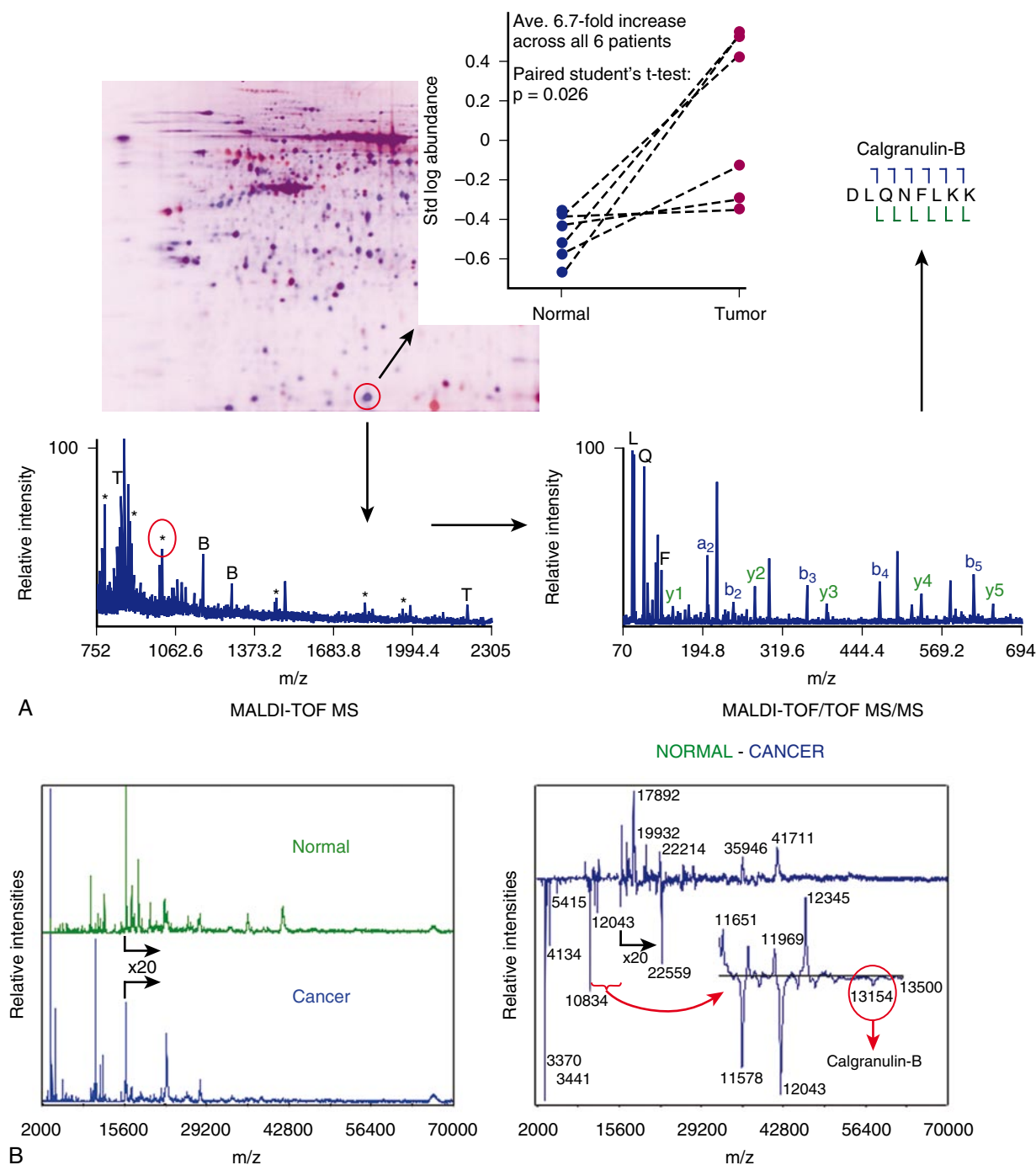


FIGURE 22-7 COMPLEMENTARITY BETWEEN DIFFERENT PROTEOMIC PLATFORMS. A: Six normal and six cancerous human resected colon biopsies samples were investigated by two-dimensional (2D)-difference gel electrophoresis-mass spectrometry (DIGE/MS). A resolved protein at an apparent molecular weight of ≈ 13 kD was found to be systematically more abundant by a factor of 6.7-fold in all cancer samples (paired Student *t* test $p = 0.026$). After protein excision, tryptic digestion followed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) peptide mapping, MALDI-MS/MS sequencing, and database interrogation, this protein was identified as calgranulin B. **B:** Tissue sections from these same biopsies were also profiled by MALDI-MS. The difference spectrum "Normal minus Cancer" shows protein signal variations between the two samples. In particular, a protein with a molecular weight of 13,154 D was found to be systematically more abundant in all cancer samples. After protein extraction, isolation by reverse-phase-high-performance liquid chromatography (RP-HPLC) and digestion by trypsin, the resulting peptides were mapped and sequenced. After database searching, this protein was also identified as calgranulin B. Comparison of the mass ranges acquirable from these two techniques (10–200 kD for DIGE, 2–70 kD for profiling) indicates a great deal of complementarity, with enough overlap to ensure confidence in comparing protein expression changes across technology platforms.

complete analysis. Although isoelectric focusing is challenging at the extreme pH ranges, relatively few proteins are predicted to have pIs below pH 3 or above pH 11. More problematic for 2D gels is the solubilization of extremely hydrophobic proteins and the detection/identification of low-abundant proteins owing to

inherent limitations of the 2D system and recovery of in-gel-digested peptides.

All analytical platforms have fundamental limits in total protein amount before resolution is affected, and this is constrained in some sample types by the presence of a few

proteins comprising the bulk of the sample (this is most problematic in serum/plasma studies [1]). Quantitative strategies using the bottom-up shotgun-based approach nicely complement MALDI-IMS and DIGE/MS because these techniques quantify abundance changes at the peptide level directly in the mass spectrometer, offering much greater sensitivity (low femtomolar) than these other approaches, and like DIGE/MS, can identify proteins using ESI-MS/MS. However, this approach is performed at the peptide level, and many post-translational modifications or protein processing can be missed due to the fact that protein detection is now based solely on the information derived from a collection of peptides within a complex mixture.

Combinations of these technologies can provide validations through complementary information. For intact protein quantification, MALDI-IMS typically resolves approximately 300 to 1,000 signals between m/z 3,000 and 70,000, with most below 20 kD. DIGE/MS typically resolves approximately 1,500 signals between 10 and 200 kD, with most signals resolving between 20 and 150 kD. In one example (Figure 22-7), a preliminary clinical study on human colon cancer using DIGE/MS revealed many protein changes that were statistically significant across the six-patient cohort (129). In particular, a protein at ≈ 10 kD was found to be statistically more abundant in all cancer samples (average 6.7-fold increase across six patient samples using DIGE, $p = 0.026$). After spot excision and tryptic digestion, the resulting peptides were mass mapped and fragmented to retrieve sequence information and the protein identified as calgranulin B (S100 A9; Figure 22-7). MALDI-MS analysis of thin sections from the same tissues revealed information on many proteins; in particular a protein detected at m/z 13,154 was found to be more abundant in all of the cancer samples (Figure 22-7). After performing a protein extract, this protein was isolated by RP-HPLC and subsequently digested. Peptide mass mapping and fragmentation of the resulting peptides allowed unambiguous identification of the protein calgranulin B.

Perspectives: Mass Spectrometry from Bench top to Bedside

The genomic and proteomic expression in cancer is enormously complex, and research investigators are eager to incorporate new tools and methodologies that provide new information to help understand this molecular complexity. Mass spectrometry brings new and extraordinarily powerful capabilities for the molecular identification of biologic pathways and processes involved in disease diagnosis, disease progression, prognosis and insights into molecular targets for therapy, and development of new drugs (145). In the early stages of tumor formation, protein patterns are complex and shift rapidly, and new molecular tools are needed to identify not only the changes in these patterns, but also identify specific proteins involved. These proteins represent potential tumor-specific markers and drug targets. When taken together with other molecular analytical tools available to research investigators, it is clear that mass spectrometry takes on a leading role in this arena.

Proteins implicated as a cause or an effect of the disease can be of value in nearly every approach to management and eventually in finding a cure for the disease. Protein patterns or signatures involve the elucidation of features in the mass spectra from disease tissue that are significant aid for the diagnosis, assessment of disease progression, choice of therapy, and correlation with survival. Beyond this, identification of specific proteins in these patterns may produce biomarkers that can be used to monitor and assess care and outcome. Some of these markers might be used in tests developed using other technologies, such as enzyme-linked immunosorbent assay or other spectroscopy-based tests. The assessment of the presence and amounts of a suite of such markers may be the next domain for mass spectrometry itself, offering wide molecular specificity and sensitivity without the need for target-specific reagents. Mass spectrometry offers a unique high-accuracy molecular specificity that will be invaluable in understanding the molecular events in carcinogenesis and will provide a unique insight into disease processes.

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Imaging and Cancer

Noninvasive imaging plays an important role in many aspects of clinical oncology, including tumor detection, differentiation between benign and malignant tumors, staging of disease, treatment follow-up, prediction of response to a particular treatment, and development of novel therapeutics. Cancer imaging can be categorized into anatomic, physiologic, and molecular imaging. Anatomic images, primarily obtained using x-ray computerized tomography (CT), magnetic resonance imaging (MRI), or ultrasound (US), can be used for tumor detection and determination of tumor size. Images that provide physiologic information such as tumor blood perfusion can be obtained using contrast enhanced x-ray CT and MRI, as well as with radionuclide imaging modalities, such as positron emission tomography (PET) and single-photon emission computerized tomography (SPECT). Molecular imaging, which involves using molecular imaging probes to detect biologic molecules in living subjects, can be performed with PET, SPECT, MRI, US, and optical imaging modalities. [Figure 23-1](#) shows clinical and preclinical imaging instruments with sample images obtained from each. This chapter will first describe the major imaging modalities that are being used in clinical oncology and cancer research. Description of major molecular imaging probes and contrast agents will then follow. Finally, we have discussed the applications of imaging in the development of therapeutic agents for cancer and future applications of imaging in oncology.

Imaging Technologies

X-Ray Computerized Tomography

X-ray CT is primarily an anatomic imaging modality. CT image contrast is dependent on tissue density, which determines the amount of x-ray beams that will be attenuated as they pass through the object of interest. In other words, at energies used for CT, imaging attenuation depends on number of electrons per unit mass or NZ/A (N = Avogadro's number, Z = atomic number, and A = atomic mass). The CT detectors measure the attenuation coefficient (μ) between the x-ray tube and detectors, which is a measure of how much x-rays are absorbed. The gray levels in a CT image represent attenuation in each voxel of a tomographic image and are expressed as Hounsfield units (HU). Bones or areas of calcification are the most attenuating and have a value of

+1,000 HU; whereas air is least attenuating and air-filled areas have a value of -1,000 HU. CT images are usually reconstructed through the mathematical process of filtered back projection. Clinical CT scanners with up to 64 detector rows are available that can acquire 64 slices at a time and spiral scanning allows acquisition of whole-body images within a few seconds ([1](#)). CT offers one of the best, combined temporal (<100 ms) and spatial (<0.05 mm) resolutions of all tomographic modalities. Rapid three-dimensional (3D) acquisition allows freezing of respiratory and bowel motion, resulting in clear images of the thorax and abdomen. CT can also provide information on tumor vasculature and blood-brain barrier (BBB) disruption by using iodinated contrast agents. The increased x-ray attenuation is proportional to the concentration of iodine (approximately 25 HU/mL). Using two-compartment models with intravascular and extravascular components, CT can measure perfusion, relative blood volume, vascular permeability, and relative extravascular volume. Contrast-enhanced CT can distinguish between malignant and benign tumors as well as between high- and low-grade tumors because of more intense and heterogeneous angiogenesis in high-grade tumors. Oral contrast agents can be used to detect gastrointestinal (GI) tract lesions. Rapid scanning enables acquisition of an entire image during the peak of the intravenous bolus contrast, optimizing the use of contrast agents and making 3D CT angiography possible ([1](#)). Because CT scanners are widely available and images between scanners are comparable, CT examinations are also used for screening, and CT is one of the most frequently used imaging modalities for assessment of cancer. However, CT scans expose patients to ionizing radiation, which limits pediatric applications of CT and the number of times an adult can be scanned in a short period of time. Finally, combined PET/CT imaging increases sensitivity and specificity of detection in many types of cancer ([2](#)) and is increasingly being used in the management of cancer patients.

Magnetic Resonance Imaging

MRI is primarily an anatomic or physiologic imaging modality in current oncologic clinical applications, but its applications has been expanded to molecular imaging in research. Anatomic MRI imaging offers the advantage of superior soft-tissue contrast and absence of bone artifacts over CT. The physics behind MRI has been explained in detail in many books and reviews ([3](#)). The imaging

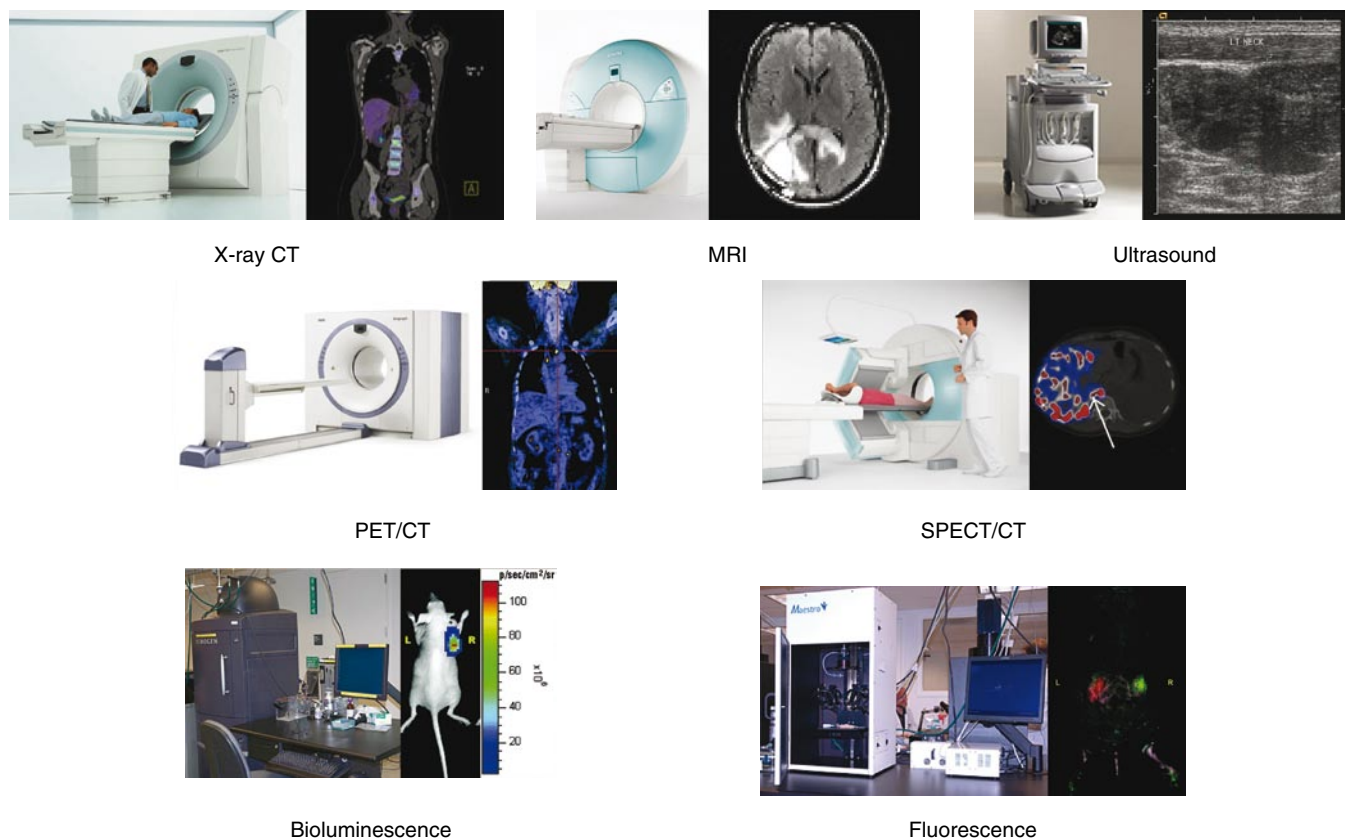


FIGURE 23-1 Clinical and preclinical imaging instrumentation and examples of oncologic images acquired using each technology.

concept is illustrated in Figure 23-2. MRI scans are T1-, T2-, proton density-, or diffusion-weighted. T1, the spin-lattice relaxation time, is the time required for protons in a constant magnetic field to relax once excited by radio frequency (RF) energy, by giving off energy to surrounding structure (the lattice). T2 is the spin-spin relaxation time required for giving energy to neighboring protons. Proton density reflects quantity of mobile protons and diffusion relates to the thermal (random) motion of protons. These scan weights are determined mainly by two operator-selected time parameters, repetition time (TR) and echo time (TE). TR is the time between RF excitations, and TE is the time between RF excitation and MR signal acquisition. Decreased T1 or increased T2 result in greater signal intensity (whiter regions) in T1- or T2-weighted images, respectively. MR signal location is determined by a frequency gradient and Fourier transform is used to compute signal amplitude at a certain frequency. Several new techniques can improve the quality of information gathered from MRI scans (4). The use of a high-field strength magnet increases signal-to-noise ratio, though it also increases heating deposition in the patient. The use of high-performance gradients can yield thinner slices with better spatial resolution. T2-weighted fluid attenuation inversion recovery (FLAIR) MR sequences can be used to improve lesion detection near cerebral ventricles and convexities (4). Dynamic contrast-enhanced (DCE)-MRI are rapidly acquired T1-weighted acquisitions, before, during, and after gadolinium

(Gd) chelate administration and can be used to distinguish radiation-induced necrosis from recurrent tumors in the brain. Since changes on DCE-MRI directly relate to changes in vascular permeability (5), it may also be used to monitor efficacy of drugs used against angiogenesis. Dynamic susceptibility-contrast (DSC) approaches are based on rapid imaging of the first pass of Gd contrast agent, inducing substantial T2 shortening, which results at first in loss and then recovery of signal in the tumor bed (6). DCE and DSC can be used to measure relative cerebral blood volume and determine tumor grade (6). Diffusion-weighted imaging may allow grading of tumor cellularity, because tumors that are more cellular have less apparent water diffusion coefficient (ADC_w). Less cellular tumors generally respond more positively to therapy (6). An early increase in tumor ADC_w has been observed on effective therapy; thus diffusion-weighted MRI is useful for monitoring response to chemotherapy (7). Diffusion MRI can also differentiate between necrotic and healthy tumor volumes (7). Functional MRI (fMRI) can be used for blood oxygen level determination (BOLD) by detecting changes in deoxyhemoglobin/oxyhemoglobin ratios in T2-weighted images. BOLD fMRI can be used to determine whether a brain tumor is close to an important functional center and thereby aid in modifying surgical approaches. Molecular imaging applications of MRI have been expanding significantly with the development of targeted contrast agents and will be discussed in more detail later in this chapter.

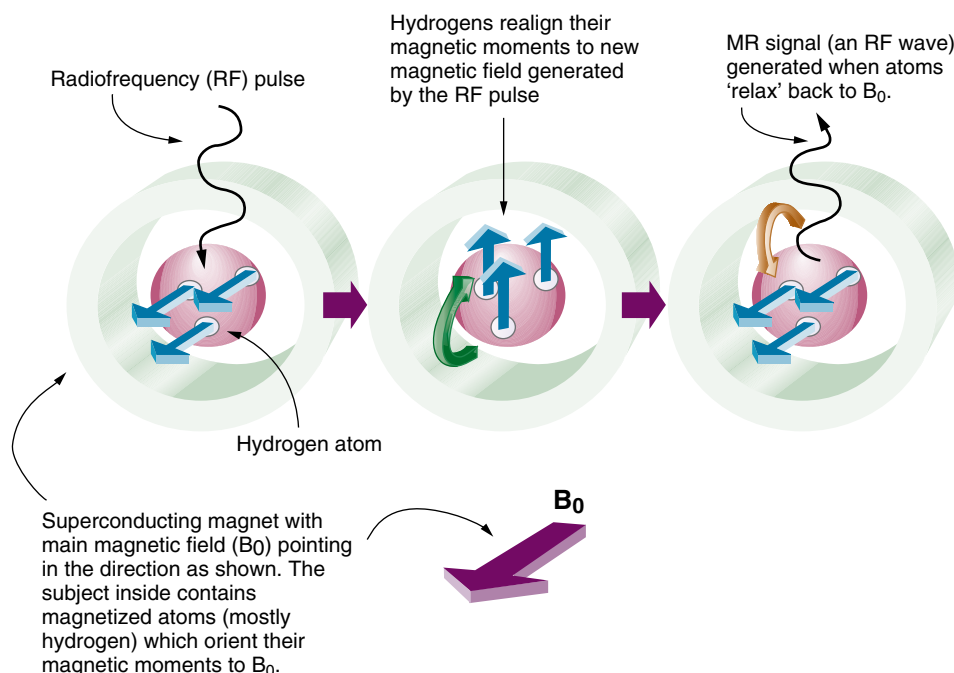


FIGURE 23-2 BASIC PRINCIPLES OF MAGNETIC RESONANCE IMAGING (MRI). In MRI, subjects are placed in a strong, external magnetic field, B_0 , produced by a hollow, cylindrical magnet. The B_0 field is nearly uniform and points parallel to the long axis of the magnet. Imaging with MRI is dependent on atomic nuclei with an odd number of protons, such as hydrogen (^1H). Such atoms have their own net magnetic field (i.e., magnetic dipole moment [MDM]) and their moments align accordingly when placed in this external magnetic field. Once equilibrium has been achieved between the subject and the magnet, energy can be added to the system in the form of a radiofrequency (RF) pulse. In most cases, this pulse, which generates its own magnetic field, can change the alignment of the hydrogen atoms such that their moments are now perpendicular to B_0 . Once the RF pulse is “turned off,” the hydrogen atoms realign or “relax” to B_0 and give up energy in the form of RF waves during the relaxation period. Receivers located in the magnet capture this RF wave. One of the calculations made from the captured information is the rate at which the hydrogen atoms relax to equilibrium. Image construction and image contrast are possible with MRI because hydrogen atoms associated with macromolecules like fat and proteins have a significantly different relaxation rate than the hydrogen atoms of bulk water. The measurements of relaxation rates can be converted into a value, which translates into image pixel value, with each pixel representing a small, representative, unit volume of the subject (voxel). On a certain MRI protocol called a T1-weighted sequence, a voxel composed mostly of fatty (hydrocarbons) protons will have a high (bright) signal since the rate of relaxation is rapid. In contrast, voxels that contain a large number of water protons, this voxel will have a low (dark) signal on T1-weighted MR imaging since the rate of relaxation is much longer. (From Biswal S, Gambhir SS. In: Templeton NS, Lasic DD (eds.). *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, 2nd ed. New York: Marcel Dekker, 2003:447–480, with permission.)

Radionuclide Imaging Modalities

PET (Figure 23-3) and SPECT (Figure 23-4) are primarily molecular imaging modalities, but can also be used for physiologic imaging (e.g., to assess blood flow/perfusion). Unlike x-ray CT and MR images, which are directly dependent on plain or contrast-enhanced tissue characteristics, radionuclide imaging tracks the biodistribution and regional concentration of a radioisotope. The radioisotopes are often covalently bound to molecules that have a specific target or will get trapped in proportion to blood flow for perfusion assessment. In radionuclide imaging, the radioactive probes (also called tracers) at first distribute nonspecifically in tissues (although they may not enter certain tissues) and then accumulate at their target sites while clearing from other sites (although they may accumulate nonspecifically in organs that are in their clearance pathway). Often, millions of cells in close proximity accumulate sufficient levels of tracer to visualize them relative to background. The SPECT radioisotopes are γ emitters and the PET radioisotopes are positron emitters. Upon collision with electrons, positrons annihilate, emitting two simultaneous γ rays that are approximately 180 degrees apart and have energies of 511 KeV each. Therefore, PET instruments contain detectors that are operated in a coincidence mode. This property gives PET advantages

such as higher spatial resolution with greater sensitivity and more quantitative images. However, SPECT scans can be done using several distinctly radiolabeled tracers simultaneously, with γ rays of different energies, something not possible with PET imaging. Relative to MRI, PET is a more sensitive molecular imaging modality and requires much fewer probe molecules, which also lessens the probability of pharmacologic effects. Clinical popularity of PET has increased with the availability of PET/CT scanners, mainly due to the increased ability for precise localization (2). In addition, PET/CT also has better attenuation correction, yielding more accurate radioactivity measurements and faster data acquisitions resulting in more efficient use of fast-decaying PET radiopharmaceuticals (2). Applications of PET and SPECT in cancer have been described in many publications and will be discussed further in this chapter (8–10).

Optical

Bioluminescence and fluorescence optical imaging are molecular imaging modalities (Figure 23-5), with applications limited to pre-clinical cancer research in small animals. Bioluminescence results from emission of photons during catalytic conversion of a probe

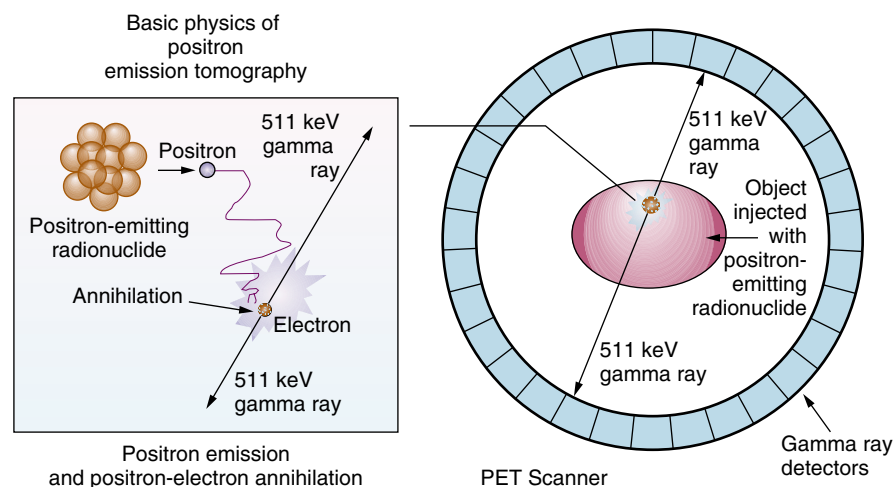


FIGURE 23-3 BASIC PRINCIPLES OF POSITRON EMISSION TOMOGRAPHY (PET) IMAGING. Molecules radiolabeled with positron-emitting isotopes may be useful as PET tracers/probes. The isotope decays by emitting a positron from its nucleus. This positron eventually collides with a nearby electron resulting in an annihilation event where two 511,000-eV photons in the form γ rays are emitted ~ 180 degrees apart. The two emitted photons travel extracorporeally and are detected nearly simultaneously as they interact with a ring of detectors (composed of scintillation crystals and photomultiplier tubes) surrounding the subject. Detection of a single annihilation event results in the “activation” of detectors opposing one another, which is recorded as a “coincident event.” The recording of multiple detector pair combinations yields a large number of these coincident lines. Sophisticated mathematical analyses of the coincident lines, which include filtered back projection and attenuation correction, yields the location of cell populations or tissues that contain the molecule labeled with the positron emitter. Tomographic images of relative probe concentration can be reconstructed in the conventional sagittal, coronal, and transverse imaging planes or in any arbitrary plane. The resultant image depicts the distribution and concentration of the radiolabeled tracer. Sensitivity of PET is in the range of 10^{-11} to 10^{-12} M/L and is independent of the location depth of the tracer of interest. It is important to note that all positron-emitting radioisotopes produce two γ rays of the same energy, so if two molecular probes—each with a different positron-emitting isotope—are injected simultaneously, there is no way for the PET camera to distinguish between the two molecular probes. Therefore, to perform studies, which look at two or more distinct molecular events (e.g., suicide gene therapy and imaging apoptosis, cardiac gene therapy and perfusion ^{15}N ammonia imaging, etc.), one has to inject molecular probes separately, which allows decay of the isotope. (From Biswal S, Gambhir SS. In: Templeton NS, Lasic DD (eds.). *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, 2nd ed. New York: Marcel Dekker, 2003:447–480, with permission.)

and fluorescence is the emission of light in a certain wavelength spectrum from molecules exposed to light of different wavelengths. These photons with wavelengths between 400 and 1,000 nm are of low energies (2–3 eV; 11) and subject to significant tissue attenuation (especially due to light absorption by hemoglobin) in visible light range and lipid and water in the infrared region (12). Therefore, detection of these photons, especially emanating from bioluminescent molecules, from living subjects requires ultrasensitive cooled charge-coupled device (CCD) cameras.

Bioluminescence imaging in small animals involves luciferase reporter genes, such as Firefly (Fluc; 13), Renilla (Rluc; 14), or Gaussia (Gluc; 15) luciferases. The Fluc enzyme catalyzes adenosine triphosphate (ATP)-, Mg^{2+} -, and O_2 -dependent conversion of D-Luciferin to Oxyluciferin, emitting bioluminescence. The Rluc and Gluc enzymes catalyze ATP-independent oxidation of Coelenterazine, emitting light. Bioluminescence imaging has particularly low background signal due to inability of normal tissues to produce any signal. Fluorescent imaging agents include, near-infrared fluorochromes (NIRFs), fluorescent proteins, and quantum dots (12,16,17). NIRF and quantum dots are more suitable for small animal imaging, due to less autofluorescence above 650 nm (16). Fluorescence imaging has some background signal due to autofluorescence from normal tissues but newer strategies to minimize this signal are being used. Both fluorescent and bioluminescent imaging agents have been used to detect tumor cells through their specific molecular characteristics, metastasis or metastatic potential, apoptosis, transgene or endogenous gene expression, intracellular protein–protein interactions, and cell trafficking (11,12,18–20).

Ultrasound

The US transducer emits a high-frequency sound wave and detects its reflection. The speed at which the signal travels in a tissue, the attenuation/absorption of the wave energy by the tissue, and the features of the tissue responsible for reflection and scattering, generally subsumed in the concept of impedance, constitute the ultrasonic propagation properties of a tissue. In this manner, US provides information about separation of tissue structures and tissue contrast. Transducers control axial (along the beam) and lateral (across the beam) resolution and are the “eye” of US. The length of the US pulse determines axial resolution. Lateral resolution is defined by the width of the US beam. Increased frequency improves resolutions at the cost of reduced beam penetration due to increased attenuation. Tissue depth is estimated by the time it takes the US wave to reflect back to the transducer (assuming constant speed of sound), and signals from more distant tissues attenuate as they pass through tissues, resulting in weaker signals. Fluid-containing structures do not reflect signal and are black, whereas calcified structures reflect strongly and are white. Lower echo level is returned to the transducer from the structure behind an area of high attenuation, resulting in a shadow. An area of low attenuation enhances the echo levels behind it. Reflective and refractive bending of the US beam due to velocity differences between the fluid and surrounding tissue cause shadowing behind certain tumors that produce high attenuation and behind the edges of larger fluid-filled areas, such as cyst or gallbladder. Malignant tumors exhibit much more complex echo patterns with more heterogeneity in size and intensity of the echoes than do benign tumors.

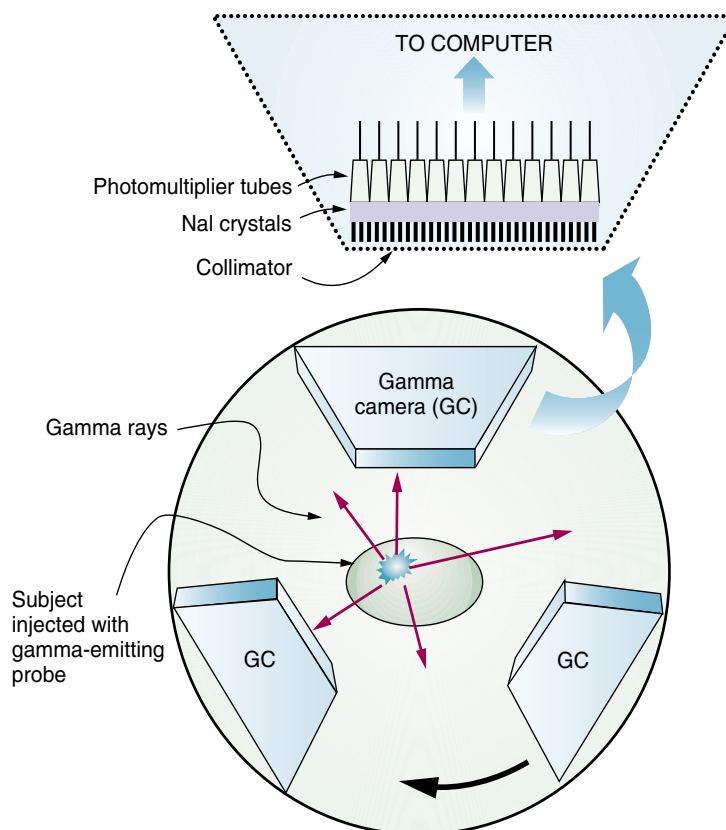


FIGURE 23-4 BASIC PRINCIPLES OF GAMMA CAMERA/SINGLE-PHOTON EMISSION COMPUTERIZED TOMOGRAPHY (SPECT) IMAGING. Imaging with a gamma camera is similar to positron emission tomography (PET), but the radiolabel emits gamma rays instead of positrons. A variety of radioisotopes, each emitting at characteristic photon energies, can be attached to a variety of molecules. Examples of isotopes include ^{111}In (171, 245 keV), ^{125}I (27–35 keV), ^{131}I (364 keV), and $^{99\text{m}}\text{Tc}$ (140 keV). Once introduced into the body, detection of these radiolabeled probes is performed with a gamma camera, a scintillation detector consisting of collimator, a sodium iodide crystal, and a set of photomultiplier tubes. Upon decay, these radionuclides emit a gamma ray at their characteristic energies in different directions. Some of the gamma rays will scatter or lose energy and others may never interact with the camera. Since the gamma camera is situated only on one side of the subject, only rays directed toward the camera will potentially be “captured.” Furthermore, only those gamma rays that arise parallel to collimator will be detected since scattered gamma rays will be absorbed by the collimator. Those rays, which successfully reach the crystal and are stopped by it, will be converted into photons of light. In turn, the photomultiplier tubes convert the light into an electrical signal that is proportional to the incidental gamma ray. Gamma rays, which have a lower than the expected characteristic energy upon arrival at the detectors, are thought to be the result of scattering and summarily rejected from the analysis. Since gamma cameras acquire data in a single plane, the resultant images are a two-dimensional representation of a three-dimensional subject (referred to as planar imaging). SPECT acquires volumetric data by rotating a gamma camera around the subject and/or using multidetector systems (shown). (From Biswal S, Gambhir SS. In: Templeton NS, Lasic DD (eds.). *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, 2nd ed. New York: Marcel Dekker, 2003:447–480, with permission.)

The advantages of US are good soft-tissue contrast, easy application, low cost, lack of ionizing radiation, and acquisition of real-time images, allowing individualized examination of almost all body regions and providing real-time guidance for biopsies. However, ultrasonography is quite operator dependent, limiting reproducibility. Also, obese people are difficult to scan and bowel gas makes it impossible to detect tumors behind gas-filled bowels (21). The primary applications of US in oncology are in the diagnosis of gynecologic tumors, detection of liver metastasis, evaluation of the kidneys, detection of enlarged para-aortic nodes, and the characterization of tumors by combining this technique with biopsy of primary tumors and of nodes in the neck, axillary, or inguinal region (21). Encapsulated microbubble contrast agents induce greater reflection of acoustic waves, increasing the echogenicity of the areas they traverse to, and enhance the gray-scale or Doppler image. Furthermore, US can be applied for therapeutic purposes, such as heat coagulation of tissues and ways to improve drug delivery (22).

Other Imaging Modalities

Conventional x-rays are widely available but provide limited information compared with other anatomic imaging modalities due to projection of all body structures in a single plane and very low inherent soft-tissue contrast. Due to good contrast between fat, tumor, and calcification, conventional x-ray is widely applied for screening breast tumors (mammography). Otherwise, initial diagnosis of tumor with this modality is only possible in lungs and bones (21). Conventional x-ray can also be used to visualize tumors of the GI tract, using enteral contrast agents. Magnetic resonance spectroscopy (MRS) can detect metabolites, such as elevated choline in brain, breast, prostate, colon, and cervical cancers, metastasis, and decreased *N*-acetyl aspartate (the most abundant amino acid in normal brain tissue; 23). Intravital and photoacoustic microscopy can be used for high-resolution in vivo imaging (24,25). Endoscopic

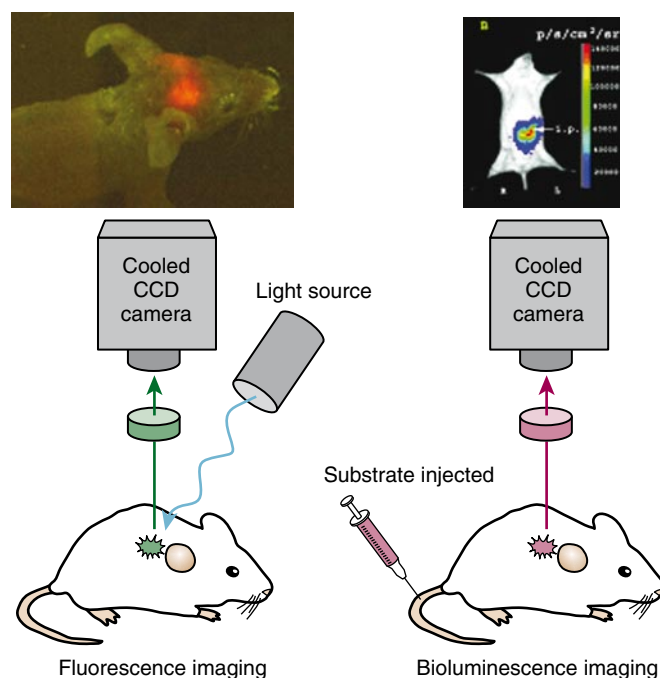


FIGURE 23-5 BASIC PRINCIPLES OF OPTICAL CHARGE-COUPLED DEVICE (CCD) IMAGING (FLUORESCENCE/BIO-LUMINESCENCE). There are fundamentally two different types of optically-based imaging systems: fluorescence imaging, which uses emitters such as green fluorescent protein (GFP), wavelength-shifted GFP mutants, red fluorescent protein (RFP), “smart” probes and near-infrared fluorescent (NIRF) probes, and bioluminescence imaging, which uses systems such as Firefly luciferase/*D-luciferin* or Renilla luciferase/*Coelenterazine*. Emission of light from fluorescent markers requires external light excitation whereas bioluminescent systems generate light de novo when the appropriate substrates/cofactors are made available. In both cases, light emitted from either system can be detected with a thermoelectrically cooled CCD camera since they emit light in the visible light range (400–700 nm) to near-infrared range (≈ 800 nm). Cooled to -120°C to -150°C , these cameras can detect weakly luminescent sources within a light-tight chamber. Being exquisitely sensitive to light, these desktop camera systems allow quantitative analysis of the data. Image shown above the *Fluorescence Imaging* schematic is a representative image obtained from a glioma model, which expresses RFP. (From Anticancer, Inc., San Diego, CA, with permission.) The method of imaging bioluminescent sources in living subjects with a CCD camera is relatively straightforward: The animal is anesthetized, subsequently injected with the substrate, and immediately placed in the light-tight chamber. A light photographic image of the animal is obtained which followed by a bioluminescence image captured by the cooled CCD camera positioned above the subject within the confines of the dark chamber. A computer subsequently superimposes the two images on one another, and relative location of luciferase activity is inferred from the composite image. An adjacent color scale confers relative concentration of luciferase activity. Sample image above the *Bioluminescence Imaging* schematic is a typical image obtained with this technology. (From Bhaumik S, Gambhir SS. *Optical imaging of Renilla luciferase reporter gene expression in living mice*. PNAS 2002;99:377–382, with permission.) In this example, image was obtained after intravenous injection of *Coelenterazine* into a mouse containing intraperitoneal *Renilla luciferase*-expressing tumor cells. Significant bioluminescence is detected from the region of the xenograft. (From Biswal S, Gambhir SS. In: Templeton NS, Lasic DD (eds.). *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, 2nd ed. New York: Marcel Dekker, 2003:447–480, with permission.)

fluorescent imaging approaches are being developed to distinguish neoplastic from normal epithelium in the esophagus and colon (26).

Imaging with Contrast Agents and Molecular Probes in Oncology

Although molecular imaging (MI), like anatomic imaging, can be helpful in cancer diagnosis and staging, it can also provide useful

information for patient treatment. For example, molecular imaging may help determine whether a tumor will respond to a certain treatment, reveal the effectiveness of treatment earlier than other mechanisms, and illustrate the pharmacokinetics of a therapeutic agent in patients with cancer. MI can be used to image tumor metabolic activities, hypoxia, apoptosis, proliferation, angiogenesis, and gene expression. Furthermore, MI is playing an expanding role in oncology research. Tables provided by El-Deiry et al. describe features and applications of MI as well criteria for development of imaging probes and a list of cancer-specific targets (26). Table 23-1 in this chapter provides a list of probes commonly used to image specific conditions caused by cancer, which will be discussed in the following sections. Figure 23-6 illustrates cellular targets of MI probes and Figure 23-7 illustrates the structures of some common MI probes. The United States National Center for Biotechnology Information (NCBI) at the National Institutes of Health (NIH) has developed a Molecular Imaging and Contrast Agent Database (MICAD) that is available online at <http://www.ncbi.nlm.nih.gov/books/bookres.fcgi/micad/home.html>. The MICAD is continuously updated with information about newly developed MI probes.

MRI Contrast Agents

MRI contrast agents can be used to determine tumor oxygenation, vascular volume, blood flow, permeability, vessel size and density, leakiness, and grade (27). MR probes have been developed for detection of specific receptors on tumor cells (28). MR contrast agents are detected by the changes they induce in water relaxation behavior. Gd shortens T1 and T2 values of tissue water, so they cause positive enhancement in T1-weighted images and negative enhancement in T2-weighted images. Liver MRI contrast agents decrease T1 and T2 relaxation times of liver parenchyma (29). Gd and manganese (Mn) agents decrease T1, so liver signal increases in T1-weighted images. Superparamagnetic iron oxides (SPIOs) decrease T2, so liver signal decreases in T2 weighted sequences. Ultrasmall SPIO (USPIO) decrease T1 and T2. Nonspecific Gd-chelates with extracellular distribution are used in liver MRI, because they are safe, inexpensive, and able to reveal other abdominal lesions. The hepatocyte-selective contrast agent mongafodipir trisodium (Mn-DPDP), is strongly paramagnetic, shortening the T1 relaxation time. The pancreas, kidneys, adrenal glands, heart muscles, and liver metastases of endocrine-originated tumors, in addition to hepatocellular tumors, also take up Mn-DPDP. Liver-selective Gd chelates can be eliminated in the urine and bile because of a benzene chain in their structure allowing them to connect to anion-carrying proteins in hepatocytes. SPIO (diameter > 50 nm) are taken up by the macrophage-monocyte cells in the liver, spleen, and bone marrow, leading to signal loss in T2*- and T2-weighted images. Monoclonal antibody (or minibody, diabody) and peptides conjugated to Gd or monomeric iron oxide nanoparticles (MIONs) have also been designed for the detection of specific protein receptors on cancer cells. These contrast agents have been discussed in detail by Artemov et al. (28) and briefly in the section on receptor targeting of this chapter.

Table 23-1 List of Imaging Probes/Contrast Agents Used in Cancer Molecular Imaging

Imaging Probes/Contrast Agents	Comments
Tumor blood flow	
Gadolinium chelates (DTPA) ⁷	Dynamic contrast-enhanced MRI reveals vascular volume with high resolution.
[¹⁵ O]-water	Short half-life (2 min) PET blood flow imaging tracer.
Tumor hypoxia	
Deoxyhemoglobin (BOLD MRI imaging of endogenous contrast) ^{7, 40}	Paramagnetic deoxyhemoglobin decreases T ₂ in T ₂ -weighted images Signal not only affected by Po ₂ , but also pH, hematocrit, and flow
[¹⁹ F]-Perfluorocarbon MRI ⁷	Monitor changes in tumor Po ₂ . Insensitive at radiobiologic hypoxic Po ₂ levels.
Radiolabeled nitroimidazole drugs ([¹⁸ F]FMISO, [^{99m} Tc]-EC-Metronidazole)	Trapped within hypoxic cells by nitroreductase enzymes, which requires cell viability.
[⁶⁴ Cu]-ATSM	Accumulates in hypoxic cells with high levels of NADH.
[¹⁸ F]-FETA or -FAZA	Potentially more potent than FMISO for imaging hypoxia.
Tumor metabolism	
[¹⁸ F]-FDG	Most popular PET tracer. Accumulates in cancer cells due to increased expression of hexokinase and glucose transporters. Also accumulates in granulocytes and macrophages and renal pathways and has a high background in the brain.
[^{99m} Tc]-ECDG ³⁶	Evidence of accumulation through D-glucose metabolic pathway and higher tumor to brain and muscle ratios relative to [¹⁸ F]FDG in rodents. Needs further evaluation.
[¹¹ C]-acetate or choline	Target lipid synthesis. Radiolabeled acetate is not eliminated through the kidney's and bladder.
[¹⁸ F]-choline	
Tumor proliferation	
[¹⁸ F]-FLT	Phosphorylated by thymidine kinase 1 without being incorporated into DNA. Has high bone marrow, liver and urinary tract uptake. Better than [¹⁸ F]FDG for evaluation of brain tumors due to low brain background.
[⁶⁸ Ga]-EC-guanine	Can distinguish between tumor and inflammatory cells.
[¹⁸ F]FDG	Activity correlates with the number of viable cells.
Apoptosis	
Radiolabeled and fluorochrome labeled annexin V ^{38, 47}	Selective and high affinity binding to phosphatidylserine on apoptotic cells. Currently, for clinical imaging, [^{99m} Tc]-HYNIC-labeled annexin V is mostly used. Disadvantages: Poor pharmacokinetics of the currently conjugated annexin V. Annexin V also accumulates in necrotic cells.
Angiogenesis	
Radiolabeled Cox-2 inhibitors	Cox-2 enzyme plays an important role in angiogenesis and cancer progression.
Radiolabeled RGD peptides ([¹⁸ F]-Galacto-RGD) ³²	Detects $\alpha_v\beta_3$ integrin, which plays an important role in angiogenesis and metastasis.
Radiolabeled VEGF	Detects VEGF receptor, which is involved in angiogenesis.
Surface receptors	
[¹⁸ F]-FES	Detects estrogen receptor in breast cancer patients, predicting response to tamoxifen treatment.
[¹⁸ F]-FDHT	Detects androgen receptor in prostate cancer patients and can be used to monitor anti-androgen therapy.
[⁶⁸ Ga]- or [¹⁸ F]-labeled sst2 ligands	To detect sst2-positive tumors and identify patients suitable for [⁹⁰ Y]DOTATOC and [¹⁷⁷ Lu]Octreotate radiotherapy.
[^{99m} Tc]-MDP	Accumulates in areas of active bone formation.
MRI contrast agent chelated monoclonal antibodies, diabodies, and minibodies ²⁸	Pretargeting methods or linear polymers can be used to deliver high numbers of Gd to target receptor. Disadvantages are restricted access of high-molecular-weight imaging agents and low sensitivity requiring high concentrations. Degraded probes may release toxic Gd.
Radiolabeled antibodies, diabodies, and minibodies	Specific imaging of carbonic anhydrase IX, CEA, Her2/Neu and EGFR has been demonstrated. Slow clearance necessitates long half-life isotopes.

(Continued)

Table 23-1 List of Imaging Probes/Contrast Agents Used in Cancer Molecular Imaging—(Continued)

Imaging Probes/Contrast Agents	Comments
Transporters	
Thallium-201	Accumulates in tumors similar to potassium ions. Mainly accumulates in viable tumors and barely in necrotic tissues. Disadvantages: Uptake is tumor type dependent and sometimes less accumulates in highly aggressive, vascular tumors.
^{99m} Tc-MIBI	Tumor uptake affected by blood flow, lipophilicity, net positive molecular charge, mitochondrial negative charge, mitochondrial density, leakage of immature tumor blood vessels and multidrug resistance P-gp activity.
[^{99m} Tc]-(V)-DMSA	Accumulation mediated by type III sodium phosphate cotransporters. Can detect tumors with intense proliferative activity; thus identify very aggressive tumors. Also useful for evaluation of chemotherapeutic agent (Etoposide) efficacy.
[¹¹ C] and [¹⁸ F] labeled AAs	AA transporters are overexpressed in tumor cells, but not inflammatory cells. Radiolabeled AA may be better than [¹⁸ F]FDG for brain tumor imaging.
[¹⁸ F]-FDG	Accumulation is partially due to increased glucose transporter expression.
Reporter gene imaging	
PET examples: HSV1-sr39tk or HSV1-tk/ [¹⁸ F]FHBG ^{52, 58, 59} and HSV1-tk/[¹²⁴ I]FIAU ⁶⁰	Probes are phosphorylated by HSV1-sr39TK or HSV1-TK enzymes; thereby trapped within cells expressing them. For probe comparison refer to Min et al. ⁶¹ For in vitro detection and imaging procedures refer to Yaghoubi et al. ^{62, 63}
Bioluminescence examples: Firefly luciferase (Fluc)/D-Luciferin ¹³ and Renilla luciferase (Rluc)/Coelenterazine ¹⁴	D-Luciferin and Coelenterazine are oxidized in cells expressing Fluc and Rluc, respectively, emitting light. Fluc/D-Luciferin reaction is ATP and Mg dependent.

AA, amino acid; ATP, adenosine triphosphate; DG, D-glucose, 2-fluoro-2-deoxy-D-glucose; FMISO, ¹⁸F-fluoromisonidazole; Gd, gadolinium; Mg, magnesium; MRI, magnetic resonance imaging; PET, positron emission tomography; VEGF, vascular endothelial growth factor.

Tumor Vasculature

Tumor micro vessels are sinusoidal, fragile, and hyperpermeable, with discontinuous basement membranes. They are also poorly differentiated, leaky, spatially heterogeneous, and lack smooth muscle cell lining. These vessels are disorderly branched, have

arteriovenous shunts, and collapse acutely and transiently and their spread lags proliferation of cancer cells, resulting in areas of hypoxia and necrosis within the tumor. Tumor perfusion can be assessed with arterial spin-labeling method (ASL), with water protons within the arterial blood pool serving as perfusion markers. However, endogenous contrast MRI methods do not provide quantitative measures of vascular volume or permeability. PET can

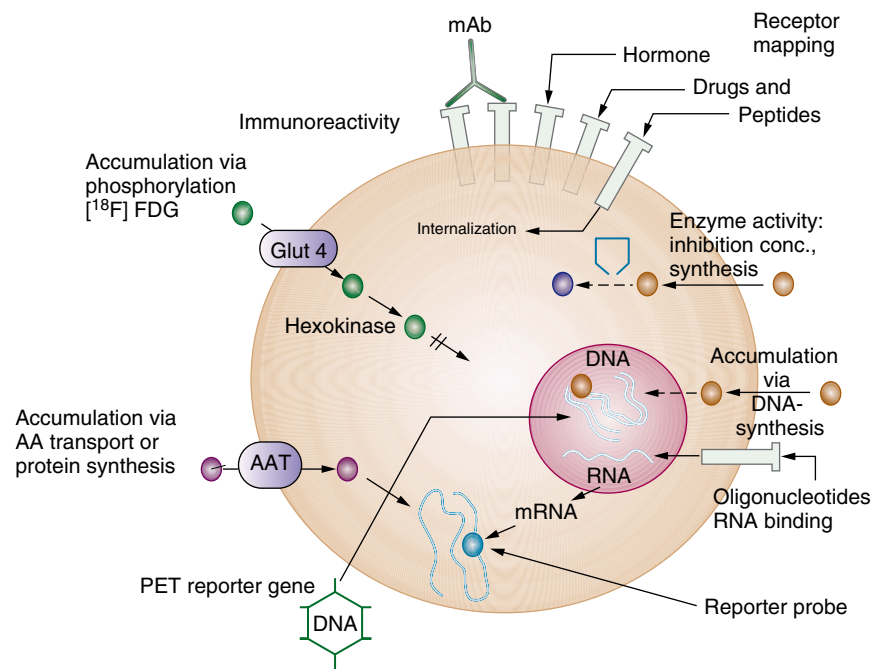


FIGURE 23-6 Cellular targets of molecular imaging probes.

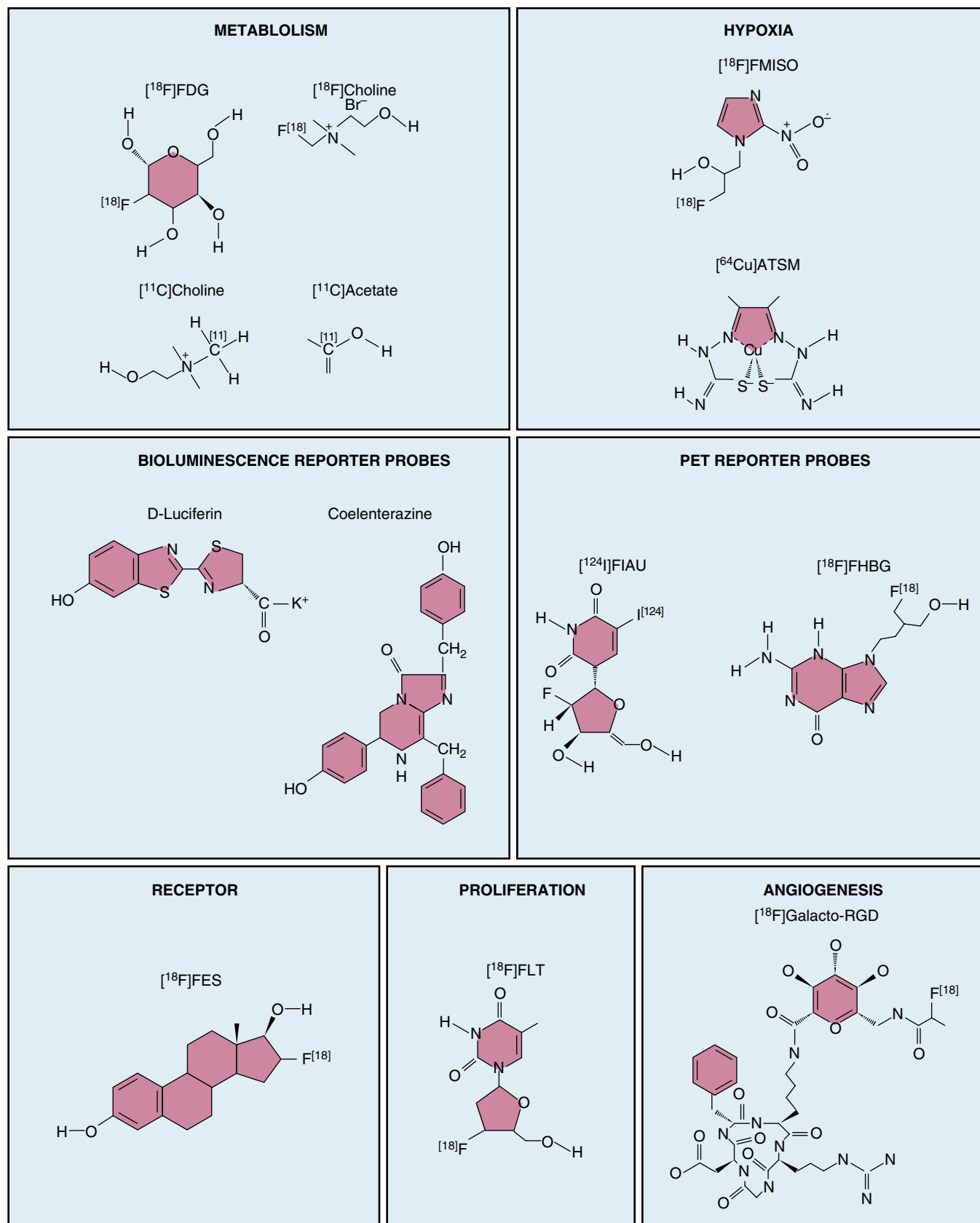


FIGURE 23-7 Chemical structures of some commonly used molecular imaging probes.

measure blood flow with [^{15}O]water, even though the 2-minute half-life of ^{15}O makes this study challenging. Monitoring angiogenesis by imaging the expression of proteins involved in tumor angiogenesis and progression is useful for determining tumor invasiveness and also response to treatment. The cyclooxygenase-2 (COX-2) enzyme plays an important role in angiogenesis and cancer progression. $^{99\text{mTc}}$, ^{18}F , and ^{11}C -radiolabeled COX-2 inhibitors have been synthesized and preliminarily evaluated for SPECT and PET imaging of COX-2 expression, but clinical imaging with these probes has not been reported (30,31). Another protein involved in tumor metastasis and angiogenesis is integrin $\alpha\beta_3$, which can be imaged with [^{18}F]Galacto-RGD (32) and other RGD derivatives (33,34). Research in imaging the VEGF receptor with radiolabeled VEGF has also been reported (35).

Metabolism

A variety of PET tracers are available for tumor metabolic imaging. The fluorine-18-labeled analogue of D-glucose, 2-fluoro-2-deoxy-D-glucose [^{18}F]FDG, is the most commonly used tracer in PET. Tumors satisfy their energy requirements, mainly through the glycolytic pathway, and often have increased expression and activity of glucose transporters and hexokinase; thus [^{18}F]FDG accumulation may be significantly increased in metabolically active cancer cells. Interpreting [^{18}F]FDG PET images may be complicated due to its accumulation in activated inflammatory cells such as granulocytes and macrophages and in the renal collecting system due to urinary excretion of the tracer. ^{68}Ga -EC-glucosamine imaging of glucose transporter has been shown to differentiate tumor and inflammation better than [^{18}F]FDG in mouse tumors (30); however, this strategy is in need of much more validation. [$^{99\text{mTc}}$]ethylenedicysteine-deoxyglucose (ECDG) is an SPECT analogue of [^{18}F]FDG that can be used for tumor detection (36), but whether its imaging mechanism is similar to [^{18}F]FDG will need to be further investigated. Glucose uptake and hexokinase activity may be low in some slow growing cancers, such as androgen dependent prostate cancer. Furthermore, detection of brain tumors is difficult, due to high uptake of [^{18}F]FDG in normal brain tissue. Carbon-11-labeled metabolic tracers, such as acetate or choline may be used to overcome the problem of background in and around kidneys and bladder. Both of these tracers target lipid synthesis. Acetate is converted to acetyl-coenzyme A in the mitochondria and since the radiolabel is on the -COOH group, it is released as $^{11}\text{CO}_2$, which gets diluted throughout the body in the bicarbonate pool or expired, thus does not accumulate in the kidneys or bladder. Choline is necessary for phospholipid synthesis in cell membranes, cholinergic neurotransmission, transmembrane signaling, cholesterol transport, and metabolism. Choline is phosphorylated to phosphatidylcholine, which accumulates during the S phase of the cell cycle and is required for the formation of phospholipid membranes. Choline-depleted cells arrest at the G_1 phase of the cell cycle. Tumor cells have negligible de novo choline synthesis, but phosphorylcholine is found at relatively high levels in cancerous tissues compared with normal tissues (37). Therefore, imaging with radiolabeled choline may measure proliferative activity. [^{18}F]-

choline has prompt urinary excretion (37), thus not alleviating the problem of high urinary bladder background. In addition, [^{18}F]-choline accumulates highly in inflammatory tissues (38).

Proliferation

High proliferation rates require increased DNA synthesis; thus imaging of nucleotide analogues can provide a direct measure of proliferation activity. Because only nucleotides containing the thymidine base incorporate exclusively into DNA, mostly radiolabeled analogues of thymidine have been tried for imaging DNA synthesis. ^{18}F -labeled thymidine, the most promising for imaging proliferation, is phosphorylated by thymidine kinase-1 (TK1), but not incorporated into DNA. TK1 expression is induced at the S phase, then declines at the G_2 phase. The 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT) accumulates in tumors, bone marrow, and urinary tract and liver (due to glucuronidation) before being excreted. [^{18}F]FDG uptake is also an indicator of tumor proliferative activity and is strongly correlated with the number of viable cells. ^{68}Ga -EC-guanine may be useful as a tumor-proliferation imaging agent and can distinguish between tumor and inflammation. Because [^{18}F]FLT does not accumulate significantly in normal brain tissue and prostate tumors have low glucose utilization, it may be more suitable than [^{18}F]FDG for the evaluation of prostate and brain tumors. For a more detailed discussion of tumor proliferation imaging agents refer to a review by Shields (39).

Oxygen Availability

Hypoxic tumors are often resistant to traditional radio- and chemotherapy, resulting in recurrence. Hypoxia also induces angiogenesis and increases the chances of metastasis by cancer cells. Clinical imaging of hypoxia is possible using MRI, PET, and SPECT (7,38). Deoxyhemoglobin (dHb) can act as an endogenous contrast agent, causing blood vessels to appear darker in T2-weighted MRI images (BOLD imaging method). Nitroimidazoles accumulate in hypoxic cells after nitro-reductase catalyzed transformation to reactive oxygen radicals that bind to macromolecular cellular components. The transformation is oxygen reversible, reducing accumulation in normoxic cells, and the requirement for active enzymes also prevents accumulation in necrotic cells. ^{18}F -fluoromisonidazole (FMISO) is one of the most popular of these compounds for imaging hypoxia in different human tumors, although, FMISO has a slow clearance, requiring 2 to 3 hours to reach sufficiently reduced background, which exceeds the half-life of ^{18}F . $^{99\text{mTc}}$ -EC-metronidazole can also be used as a SPECT hypoxic marker. Another PET tracer, ^{64}Cu -labeled methylthiosemicarbazone (ATSM) is distinct from FMISO in that it accumulates in cells with high levels of reducing molecules, such as NADH, as a consequence of hypoxia. Still, additional preclinical and clinical trials are needed to compare FMISO, ^{64}Cu -ATSM and newer hypoxia imaging agents such as [^{18}F]Fluoroetanidazole (FETA) and [^{18}F]Fluoroazomycin-arabinofuranoside (FAZA) for imaging

hypoxia in tumors. Padhani et al. (40) have reviewed different hypoxia imaging techniques, applications of hypoxia imaging and the challenges of hypoxia imaging in more detail.

Receptors and Antigens

Molecular imaging of cancer cell-specific surface receptors with a small molecule, peptide, or antibody probe may not only have diagnostic applications, but may assist in choosing the most suitable treatment regimen. Because of higher imaging sensitivity and easier access to cell surface receptors, radionuclide imaging probes have thus far shown greater potential for imaging patients than receptor-targeted MRI probes. 16α -[^{18}F]fluoroestradiol- 17β (FES) PET has been used for imaging estrogen receptors in patients with breast cancer and can help predict response to tamoxifen treatment (41). In prostate cancer, androgen receptors can be imaged with 16β -[^{18}F]fluoro-5-dihydrotestosterone (FDHT) and FDHT PET may be used to monitor antiandrogen therapy (41). ^{68}Ga - or ^{18}F -labeled ligands have been developed that bind somatostatin receptor subtype 2 (sst2), highly accumulating in sst2-positive tumors. These probes are useful for identifying good patient candidates for [^{90}Y]DOTATOC and [^{177}Lu]Octreotate radionuclide therapy (41). Binding of an MI probe to a molecule that makes up an organ can yield images of that organ's structure. For example biphosphonates avidly bind hydroxyapatite crystals, and [$^{99\text{m}}\text{Tc}$]methylene diphosphonate ([$^{99\text{m}}\text{Tc}$]MDP) is one of the most widely used agents for bone scintigraphy (9). [$^{99\text{m}}\text{Tc}$]MDP bone scintigraphy is also one of the most common methods for evaluating bone metastasis in patients with cancer.

Imaging with radiolabeled monoclonal antibodies (mABs), diabodies, or minibodies requires conjugation to long half-life radioisotopes, because of their slow clearance. The ^{89}Zr and ^{124}I labeled mAB of G250, membrane-associated carbonic anhydrase IX, may be useful for imaging renal cell carcinomas that are proliferating under hypoxic conditions (37). ^{64}Cu -labeled carcinoembryonic antigen and ^{131}I , ^{111}In -, and ^{64}Cu -labeled Her2/neu minibodies have been developed, demonstrating specific targeting through micro-PET imaging in mice (42–44). C225, a chimeric monoclonal antibody that inhibits growth of endothelial growth factor receptor (EGFR)-expressing tumor cells, has been conjugated to $^{99\text{m}}\text{Tc}$ -EC for imaging EGFR with SPECT (30). Antibody imaging with MRI has been complicated by the size of the contrast agents, which is required for detectability but limits their reaching the cell surface receptors. Another concern with Gd-conjugated antibodies is the release of toxic Gd, which is being addressed by evaluating more stable conjugates (28). Finally, large concentrations of receptor-targeted contrast agents may actually have a pharmacologic effect by interfering with cell signaling mediated by the receptor.

Apoptosis

Monitoring apoptosis of cancer cells following therapy may be a direct way to detect early response. Annexin V, a 36-kD

calcium-dependent phospholipid, binds with high affinity and specificity to phosphatidylserine, which is externalized to the extracellular side of the plasma membrane during the early phases of apoptosis (38). Annexin V has been labeled with $^{99\text{m}}\text{Tc}$, ^{111}In , ^{68}Ga , ^{125}I , ^{124}I , ^{123}I , and ^{18}F fluorochromes (Cy5.5) and cross-linked iron oxide for imaging of apoptosis in living subjects (38,45,46). $^{99\text{m}}\text{Tc}$ -annexin V has been evaluated for monitoring anticancer therapy in patients (38,45). The iodine-labeled annexin V is rapidly deiodinated in vivo and the long half-life of radio-iodine may not even be necessary due to the short duration of the apoptosis process. ^{18}F -annexin V may have the suitable half-life ($t_{1/2}$ = 110 minutes) and has been evaluated in small animal models of apoptosis (46). However, one of the limitations of Annexin V for imaging apoptosis is compromised specificity, since the membranes of necrotic cells become permeable and accumulate annexin V. Finally, optimal timing of apoptosis imaging needs to be determined. Annexin V imaging probes have been reviewed in detail by Boersma et al. (47).

Cellular Transporters

Malignant tumors appear to express higher levels of the Na^+/K^+ ATPase pump than benign cells and this pump is more active in metastatic cells (38). Thallium 201 (^{201}Tl) is actively transported by the Na^+/K^+ ATPase pump and to a lesser extent through the Na^+/K^+ , 2Cl^- cotransport and stored in cell cytosols. Therefore, ^{201}Tl -SPECT has been used to detect malignant tumors in the lung, thyroid, and brain, having an affinity for high grade tumors (38). Another SPECT probe that accumulates in tumor cells due to overexpression of a transporter is $^{99\text{m}}\text{Tc}$ -(V)-DMSA. $^{99\text{m}}\text{Tc}$ -(V)-DMSA uptake is specifically mediated by type III sodium-dependent phosphate transporters (NaPi cotransporters), stimulated by acidic pH (tumors have a more acidic extracellular pH than normal tissues), and inhibited by alkaline pH 9.38). $^{99\text{m}}\text{Tc}$ -(V)-DMSA is useful for detecting the relatively more aggressive and metastatic tumors (38). Also, its accumulation is decreased in apoptotic cells, due to inhibition of the type III NaPi cotransporter (38). In addition to these probes, increased accumulation of the metabolic probes, such as [^{18}F]FDG, may be due to overexpression of transporters on tumor cells. Amino acid (AA) transport is increased in malignant cells compared with normal cells, but unlike [^{18}F]FDG, their uptake is not significantly increased in inflammatory cells (38). Once transported inside the cells, AAs are incorporated into proteins or transformed into nonprotein metabolites. The appropriate AA radio-tracer has limited transformation into metabolites and limited efflux. [^{14}C]methionine, [^{14}C]tyrosine, [^{18}F]fluoroethyltyrosine, [^{18}F]fluoro-methyltyrosine, and [^{18}F]fluorodopa have been investigated for tumor imaging (41). Increased expression of the L-type system, which transports large neutral AAs across the cell membrane, appears to be responsible for enhanced uptake of these tracers (41). Because these AA tracers efficiently cross the BBB and their uptake in normal gray matter is much lower than [^{18}F]FDG, they provide improved contrast for imaging brain tumors than with [^{18}F]FDG (41).

Reporter Gene Imaging

Imaging reporter genes (RGs) provide a powerful tool for studying molecular events in living subjects (Figure 23-8; 18). They have been used to study intracellular molecular interactions (for example, protein–protein interactions; 19), image transgene expression (48), and endogenous gene expression (49), cell trafficking (e.g., metastasis or adoptively transferred cells for cancer immunotherapy; 50,51) in living subjects. With the exception of RG coding for fluorescent proteins (e.g., green fluorescent protein) the expression of RG is usually detected by specific imaging probes. The reporter probes can be activatable, emitting signal after interaction with the reporter protein, or may be emitting constant signal and detecting reporter gene expression by accumulating in tissues containing the cells that are producing the reporter proteins. Bioluminescent (Figure 23-8A), fluorescent, and some MRI RGs (Figure 23-8D) are detected by activatable reporter probes. The detection of all nuclear imaging RGs and some MRI RGs is through interactions of the probes with the reporter enzymes, receptors, or transporters, resulting in their accumulation within or on the surface of cells expressing these RGs (Figures 23-8B and 23-8C). The MRI and nuclear imaging RGs offer the greatest potential for oncologic applications in human patients. In fact, the PET RG/suicide gene herpes simplex virus-1 thymidine kinase has been imaged in cancer patients and the clinical application of PET RG is expected to expand in other gene and cell therapy trials (52); however, more research is needed to improve delivery of PET RG into target cells, such that RG expression is sufficiently high with minimal effect on the cells.

Imaging in Development of Therapeutic Strategies

Monitoring Response to Therapy

In addition to its role in diagnosis and staging of cancer, imaging can be useful in monitoring response to anticancer therapy. Anatomic imaging with CT and MRI may reveal changes in tumor size. Anticancer therapy may affect tumor blood perfusion, which can be detected by diffusion-weighted MRI and PET or SPECT imaging. Even before tumor size changes, anticancer therapy may affect the metabolic activity or proliferation rate of tumor cells. Therefore, PET tracers such as [^{18}F]FDG or [^{18}F]FLT may be useful for detecting early response. For example, significant reductions in [^{18}F]FDG uptake have been observed after treating patients with GI stromal tumors with imatinib mesylate, a protein tyrosine kinase inhibitor (53). In this case FDG-PET had detected response several weeks before CT. FDG-PET has also detected positive response to chemoradiotherapy of esophageal squamous cell carcinoma and radiotherapy of pancreatic cancers (38). A significant decrease in [^{18}F]FLT tumor uptake has also been observed in breast cancer patients after neoadjuvant chemotherapy (38). Therefore, molecular PET imaging can likely help identify nonresponders early,

prompting a change in treatment regimen. Furthermore, these imaging tracers can provide prognostic information about chances of recurrence after curative therapy (38). Patients having abnormal FDG uptakes after completion of chemotherapy or chemoradiotherapy are at a high risk of recurrence and have poor prognosis (41). However, radiation or chemotherapy may also cause an acute increase in FDG uptake (“flare phenomenon”), soon after initiation of therapy. ^{201}Tl scintigraphy can accurately monitor response to radiation treatment in patients with follicular lymphoma and uterine cervical cancers (38). It is also superior to CT and MRI in early prediction of overall survival and response to chemotherapy in patients with recurrent glioma (54). With the development of anticancer drugs that target specific molecular processes, imaging probes that provide information on the activity of those processes can also be used to monitor drug efficacy. For example, ^{11}C -choline PET imaging may assess the efficacy of choline kinase inhibitors.

Predicting Drug Resistance

Molecular imaging can be useful in predicting the expression of multidrug resistance (MDR) genes, such as P-glycoprotein (P-gp) or MDR-associated protein isoform 1 (MRP1); thereby assessing the chance of chemotherapy efficacy. P-gp is a drug efflux pump for a broad range of lipophilic molecules that include many chemotherapy drugs and MRP1 is a multispecific organic anion transporter that transports out of the cell's glutathione-conjugated drugs. These proteins may be overexpressed intrinsically or after the initiation of therapy. $^{99\text{m}}\text{Tc}$ -sestamibi ($^{99\text{m}}\text{Tc}$ -MIBI) is a lipophilic cation that can be expelled by P-gp and MRP1 in malignant cells and a negative correlation between the expression of these genes and accumulation of $^{99\text{m}}\text{Tc}$ -MIBI has been observed in several cancers (38). In malignant lymphomas and small cell lung cancer, patients with a good chemotherapeutic response had positive $^{99\text{m}}\text{Tc}$ -sestamibi scintigraphy and negative P-gp or MRP1 expression (38). $^{99\text{m}}\text{Tc}$ -MIBI may not directly image the expression of MDR genes and its intracellular accumulation also depends on proliferation, apoptosis, hypoxia, and angiogenesis. Radiolabeled antisense oligonucleotides, complementary to the MDR1 mRNA offer a potential direct method for imaging MDR1 gene expression.

Imaging Pharmacokinetics of Drugs

Molecular imaging with PET can allow analysis of drug pharmacokinetics in living subjects, because some positron-emitting isotopes, such as ^{11}C and ^{18}F , can replace their counterpart atoms commonly present in the chemical structure of most drugs. In addition, these radiolabeled drugs can be used to determine the quantity of their targets, such as protein receptors expressed on cell surfaces. Therefore, PET can be instrumental in predicting drug safety as well as efficacy. For example, the expression levels of both metalloproteinase (MMP) and $\alpha\beta_3$ integrin, which are required for tumor angiogenesis, can be imaged with their ^{11}C - or ^{18}F -radiolabeled antagonists (anti-angiogenic drugs; 38).

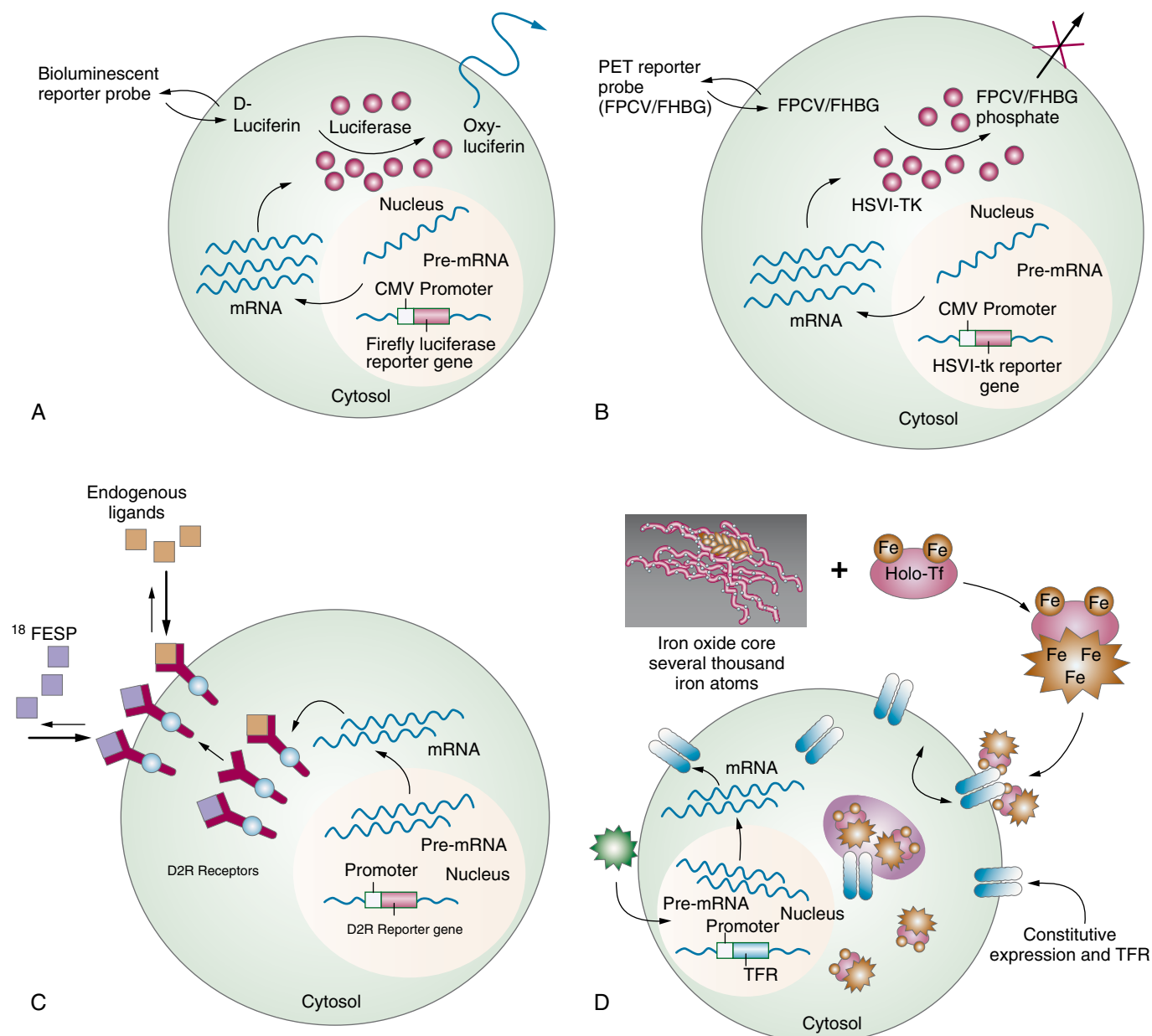


FIGURE 23-8 METHODS FOR LABELING CELLS USING REPORTER GENES. **A:** An optical reporter gene encodes for a protein that in the presence of the appropriate substrate is capable of producing light. **B:** A reporter gene that encodes for a protein that is capable of trapping a radiolabeled substrate for positron emission tomography (PET) imaging. **C:** A reporter gene that encodes for a protein receptor that is capable of trapping a radiolabeled ligand for PET imaging. **D:** A reporter gene that encodes for a receptor that is capable of transporting iron complexes into the cell that leads to detectable signal using magnetic resonance imaging.

Molecular Imaging in Cell and Gene Therapy of Cancer

The main goal of gene therapy (GT) or adoptive cellular gene therapy (ACGT) for cancer is specific and efficacious targeting. Therefore, noninvasive imaging techniques that can reveal biodistribution, time variation, and magnitude of therapeutic transgene (TG) expression or can track trafficking of therapeutic cells should be valuable for preclinical and clinical development of cancer GT and ACGT strategies. TG expression can be imaged directly with specific probes or indirectly by linking its expression to an imaging RG (48,55). Cell trafficking can be imaged by ex vivo labeling of cells with radioisotopes

(^{111}In Oxine), MRI probes (iron oxide nanoparticles), fluorochromes or quantum dots, ex vivo incorporation of imaging RG, or probes that bind specifically to a receptor on the surface of the administered cells (56,57). A variety of factors should be considered when choosing the appropriate imaging approach, including technical feasibility, desired duration of monitoring after TG or cell administration, sensitivity and specificity for a particular application, and minimal interference with the therapeutic protocol. For example, duration of cell trafficking monitoring may potentially be extended by using the RG approach or the specific probe for cell surface receptor. However, a specific cell surface receptor probe may not be available for the administered cells or RG incorporation may affect

the characteristics of some therapeutic cells. In addition to allowing the analysis of TG or therapeutic cell pharmacokinetics, imaging can be a useful tool for monitoring the therapeutic outcome noninvasively. For example, one can examine FDG uptake and degree of tumor apoptosis or proliferation before and after gene therapy as discussed in the preceding sections.

Future Outlook

Despite its rapid growth in past decade, MI is still at its infancy and many more imaging probes and imaging strategies will likely develop to expand the application of MI in oncology and other fields of medicine. Furthermore, many of the MI techniques and probes that are being applied in preclinical cancer research on research animals are likely to be optimized for translation into clinical trials. Many preclinical and clinical failures will have to occur for a small set of imaging probes to become routinely available

and reimbursed for oncology applications. Accomplishment of this goal is dependent on close collaboration among chemists, molecular biologists, biomedical instrumentation engineers, pharmacologists, translational investigators, and clinicians. These investigators will work together to develop imaging probes for newly discovered targets and enhance the sensitivity, specificity, and pharmacokinetics of probes for already characterized targets. Clinical applications of optical imaging for breast cancer, endoscopy, and intraoperative imaging are on the horizon. Development of more specific MI techniques should allow better characterization of tumor cells in diagnostic imaging, resulting in the administration of more effective treatment regimens and avoidance of unnecessary treatments. Improved MI strategies with improved in vitro diagnostics (for example serum markers) should lead to earlier detection of cancer, when therapeutic procedures are more effective. The application of MI in early assessment of response to therapy will likely grow as specific novel probes are developed to detect various molecular changes that precede anatomic or physiologic changes.

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Bioinformatics

Cancer medicine has been transformed through the elucidation of the molecular mechanisms underlying disease and its constellation of phenotypes. However, this transformation brings with it unprecedented challenges in collecting, processing, and interpreting information. The raw volume of the data associated with the comprehensive molecular characterization of disease exceeds an individual's capacity to remember and process. Moreover, an individual is simply not capable of integrating the complex and multidimensional information generated by today's technology. Interpretation of this data requires the application of information technology (IT).

An example helps illustrate this point. It is now technically possible to experimentally measure the expression state of every gene in the human genome. This has led cancer biologists to define molecular signatures that augment and may ultimately replace the classifications of cancer determined by examining cells under a microscope. But how does one extract these classifications from the 10,000s of genes measured in hundreds of samples? The most common approach uses a statistical technique called hierarchical clustering that looks for groups of samples that have similar patterns of gene expression. More specifically, correlations are calculated between the levels of RNA for the 10,000s of genes in individual samples. Samples that show high correlation are placed next to each other in a diagram called a tree. The higher the correlation, the closer the two samples (the less the distance portrayed in the tree). This is repeated over and over until all samples are placed within the tree. The tree is then inspected for the appearance of patterns that allow the investigator to suggest that there are different types of tumor represented by the branches of the tree. Additional statistical tests can be applied that demonstrate that the clusters of samples are unlikely to have occurred by chance alone. It is practically impossible to identify these patterns without the use of IT.

However, this is only the tip of the iceberg. To associate the canonical collection of genes to their expression levels requires the use of databases and mapping tools. The input to the clustering tool requires the use of additional data processing tools that adjusted for inter- and intra-experimental variation. The output of the expression experiment needs to be stored in an electronic database that permits it to be input into the clustering tool. At the end of the experiment, few investigators are satisfied with the simple observation that two or more subsets of tumors exist. They want to know

which gene differentially contributed to the differences. This requires the application of yet another set of statistical analytic tools. Once these genes are found, additional questions follow. Are these genes related to each other by family (ontology) or part of a common network (biologic pathway)? Additional electronic databases of such information need to be queried as these features are as complex in structure and numerous as the genes themselves. Do the genes have nucleotide sequence relations, protein motifs, or other common features? Reference to electronic repositories of this type of information is required to achieve these answers. Finally, does this pattern exist for other tumors of the same type, or more provocatively, for tumors of different type? Again electronic repositories of similar experiments permit one to ask and answer these questions.

It becomes obvious from even the simple problem described that the application of the tools of IT has become essential to understanding the molecular basis of cancer. The application of IT to address problems in biomedicine is called biomedical informatics. The biomedical informatics subcomponent that addresses questions of molecular etiology is called bioinformatics. Bioinformatics has become a critical technology in research investigations of the molecular basis of cancer.

The field of bioinformatics is as large and diverse as biomedicine itself. This represents both opportunity and challenge for the cancer community. A simple Google search on "bioinformatics tools" returns almost 300,000 entries (searching on "bioinformatics" and "tools" yields 5.9 million entries). There are an overwhelming number of tools, data sets, and information technology infrastructures with which one must contend.

The molecular biology dogma guides the organization and processing of experimental data. Bioinformatics is also segregated by the data and tools that support various experimental technologies. Interestingly these technologies generate orthogonal molecular characterizations that each indirectly elucidates normal and disease states. Bioinformatics provides the promise of integrating these diverse outputs to generate novel understanding.

Cancer molecular biology uses the same experimental approaches as other fields of biomedicine. However, unlike many other diseases, tumors exist as a clearly definable biologic entity that has evolved from the normal constitution of the individual in which it originates. Therefore, investigations of cancer present unique opportunities for integrated interpretation. Similarly, cancer bioinformatics is not formally distinguishable from the larger

field of bioinformatics. However, because of the novel characteristics of cancer described in the preceding paragraphs, there is an opportunity to obtain a novel coherence in cancer bioinformatics.

This chapter describes bioinformatics as applied in understanding the molecular basis of cancer. It acknowledges the convention of organizing around the experimental approaches for which the bioinformatics provide support. Bioinformatics is both analytic tools and electronically represented data. As indicated previously, tools are essential to the understanding and interpretation of the voluminous and complex data generated. Data repositories are essential for replication and re-interpretation and permit cost-efficient repurposing of invaluable data. Data repositories also serve as electronic reference materials and permit definition of biologic context for future experiments. Both data repositories and analysis tools are covered in this chapter.

Pragmatically, rather than being comprehensive, this chapter provides illustrative examples and access electronic addresses for key resources. Bioinformatics, like the rest of cancer research, is rapidly evolving. Versions of software, data resources, and infrastructures emerge much more rapidly than written material. As such, the chapter points to the Internet sites from where the resources can be accessed and to clearinghouse Internet sites where inventories are maintained.

As suggested previously, cancer bioinformatics has the opportunity to come together in a novel coherence. With agreement within the community to describe data in standard ways and to utilize common infrastructure, disparate data and interpretations can be integrated synergistically. The cancer bioinformatics community has undertaken such an effort to use common standards and infrastructure, the **cancer Biomedical Informatics Grid (caBIG)**. As such, this chapter will highlight data resources and applications that abide by the caBIG communities' conventions where they are available.

Data Resources

High-throughput laboratory technologies generate tremendous volumes of data. This represents challenges for an individual laboratory in terms of management. These large data sets also represent valuable resources for the broader cancer community. They represent reference resources to be used for comparison in future investigations. Their comprehensiveness and complexity also mean they represent rich sources for future data mining and integration. Pragmatically, the expense associated with their generation makes it critical that they are used to maximal effectiveness and are not unnecessarily duplicated.

General Portals

As implied in the preceding sections, the number and diversity of data resources in bioinformatics are quite large. The proliferation of individual laboratory Internet sites and the posting of data as supplemental material make it difficult to maintain an inventory of data resources. Interestingly, one of the most powerful tools for identifying these resources is the use of the **Google** search engine (www.google.com). The Google tool set provides powerful search capabilities to access information distributed throughout

the Internet (or on one's own computer through Google's desktop search client www.google.com/desktop). This search engine and other broad-based Internet search engines index the literature, data portals, and miscellaneous institutional and organizational Web sites that post data for reuse.

There are a number of general bioinformatics Web portals that provide access to diverse bioinformatics data. The largest and currently most heavily used is the **Entrez** resource provided by the National Institutes of Health's (NIH) National Library of Medicine's (NLM) National Center for Biotechnology Information's (NCBI) Entrez environment (www.ncbi.nlm.nih.gov/Entrez). NCBI's Entrez allows access to diverse collection of bioinformatics resources (Figure 24-1). The European Bioinformatics Institute (EBI) also provides a rich collection of bioinformatics resources (www.ebi.ac.uk).

The National Cancer Institute's (NCI) Center for Bioinformatics (NCICB) provides a cancer-focused bioinformatics portal through the Cancer Genome Anatomy Project's (CGAP) Web site (<http://cgap.nci.nih.gov>). As suggested by its title, CGAP provides genome-oriented reference data (and tools that support



FIGURE 24-1 NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (NCBI) BIOINFORMATICS RESOURCES. Shown here is the vast collection of electronic resources accessible to the biomedical community through NCBI's information retrieval infrastructure, Entrez. These resources span basic information on Mendelian disorders in humans and mouse (OMIM, OMIA), chemical structures of small molecules (PubChem), to journal indexed in PubMed.

its use). It provides reference data for genes, tissues, pathways, and chromosomes in a cancer context. Within these sections one can obtain information on cytogenetic alterations in cancer, RNAi resources, and gene expression reference data sets. The inventory of CGAP resources are shown in Figure 24-2. CGAP resources are also available through caBIG programmatic interfaces via the cancer Bioinformatics Infrastructure Objects (caBIO) services (see following sections).

An example of the CGAP's cancer data resources is its Serial Analysis of Gene Expression (SAGE) data base and the accompanying integrated tools, **SAGE Genie**, that facilitate its access and interpretation. SAGE Genie is an intuitive, visual display of human and mouse gene expression using SAGE tags (10 or 17 nucleotides in length) that have been mapped to known genes. One SAGE Genie tool, the **SAGE Anatomic Viewer** visually displays the relative expression of a given gene in normal and malignant tissues of the human body (Figure 24-3). An alternative graphic display tool, the **Digital Northern**, creates an image that approximates the output of RNA gene expression experiment and shows the relative expression of the gene in various tissues sources. Another SAGE analysis tool, the **SAGE Digital Gene Expression Displayer** (DGED) distinguishes significant differences in gene expression profiles between two pools of SAGE characterized RNA samples. The **SAGE Experimental Viewer** provides DGED results for preset pairs of samples, one under control and the other under experimental conditions. The CGAP site also permits the downloading of files of genes, tags, datasets, and gene mappings.

Specialized Resources

In addition to general portals, there are specialized data resources focused on specific types of data. It is impractical to catalog them all in this section. Instead key resources of general use or of specific interest to cancer researchers will be highlighted.

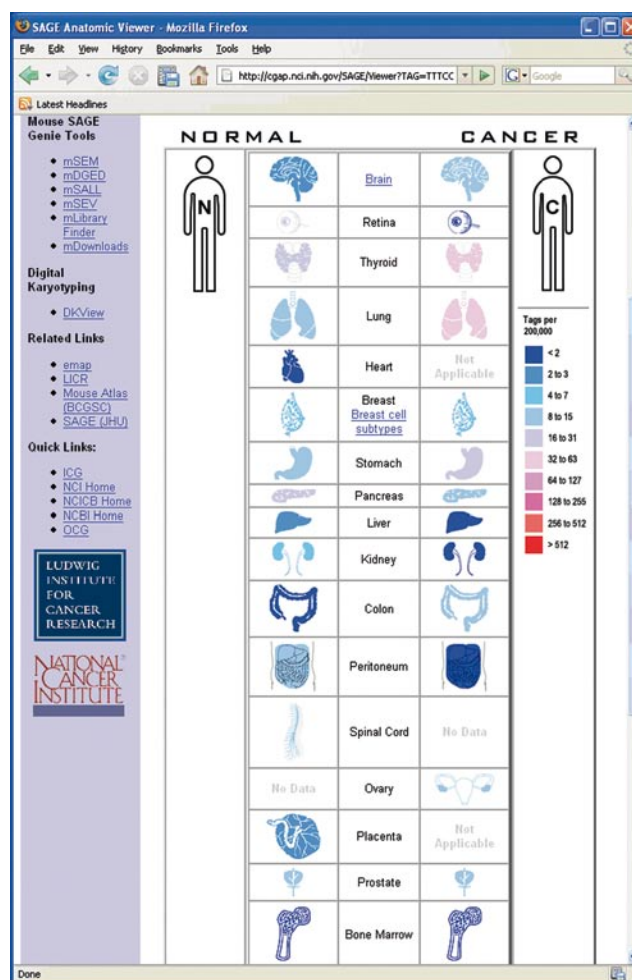


FIGURE 24-3 Cancer Genome Anatomy Project (CGAP) tool showing anatomical distribution of VEGF gene expression using Serial Analysis of Gene Expression (SAGE) Genie tool.

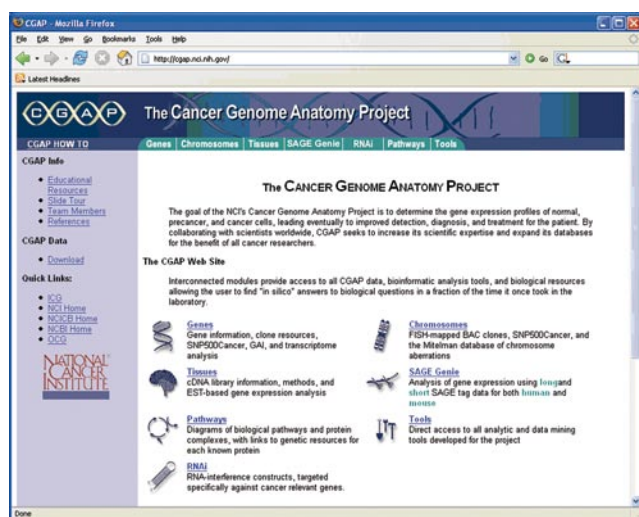


FIGURE 24-2 **CANCER GENOME ANATOMY PROJECT (CGAP) HOME PAGE.** Shown here is the World Wide Web access portal to cancer-specific molecular biologic information. Through the CGAP portal one can identify diverse information ranging from genes differentially expressed in cancer (the Genes section), compare gene expression profiles by tissue (Tissue and SAGE Genie), and cytogenetic aberrations observed in cancer (Chromosomes).

University of California Santa Cruz Genome Browser

Although there are many resources that focus on genomics, few have a cancer orientation. A genome resource not described in the preceding paragraphs is the University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu>). This browser displays information in the context of the human genome by the user selecting "tracts" to include or exclude. Tracts available are numerous and diverse and include characteristics of the human genome, observed transcripts, and alignments with other sequenced organisms. The UCSC Browser allows its users not only to display publicly accessible data, but also to display tracts of data generated by an individual investigator.

Gene Expression Omnibus

The Gene Expression Omnibus (GEO) data resource (www.ncbi.nlm.nih.gov/geo) includes microarray-based experiments measuring mRNA, genomic DNA, and protein abundance, as well as nonarray techniques such as SAGE and mass spectrometry proteomic data. GEO data can be retrieved in multiple ways including data set reference, queries of features of data sets, and specific gene

sets (referred to as Entrez GEO Profiles). GEO data can be viewed and downloaded in multiple formats. Although has the largest volume of data and the most diverse collection, there is a great deal of heterogeneity in the actual data available for any given experiment. In general, there is only minimal clinical/phenotype data and what data is present is only loosely structured. A significant fraction of data present in the resource does not contain primary instrument outputs (for example, .cel files from Affymetrix GeneChip experiments). Data can be submitted to and retrieved from the resource.

ArrayExpress

ArrayExpress (www.ebi.ac.uk/arrayexpress) is a public repository for microarray data supported by the EBI. ArrayExpress enforces the Minimal Information Annotating a Microarray Experiment (MIAME) data standards generated by the MGED (Microarray Gene Expression Data) consortium. It supports both user submission and download of array-based data. A unique feature of ArrayExpress is its storage of gene expression profiles from a curated subset of experiments in the repository.

caArray

The NCICB's microarray repository, caArray (<https://caArray.nci.nih.gov>), consists of a microarray database and microarray data analysis and visualization tools that connect directly to the data resources through caBIG-defined application programming interfaces (APIs). caArray is open-source/open-access resource with source code and APIs available in from caArray Web site. Open source means that the software is available in the form of the programming language in which it was generated. This permits its basic functionality to be read and understood by a computer programmer and to be modified in the future. Open access means the application is available without restriction to any user (academic, commercial, government, etc.). The NCICB supports public access to a working version (an "instance") of caArray to facilitate centralized data sharing. Alternatively, caArray can be used as a local microarray database that can be joined to the caBIG data federation and accessed through caGrid (see following sections).

The caArray database is MIAME compliant and allows submission of MIAME 1.1 level annotations and microarray data via Web-based submission forms. A notable feature is the ability to import MicroArray Gene Expression–Markup Language (MAGE-ML) documents using the caArray MAGE-ML Loader (caAMEL) interface. MAGE-ML is a standard computer file format for sharing data generated through a variety of microarray experimental approaches. The caAMEL application allows users to load MAGE-ML documents into the selected caArray instance. Public users may also use caAMEL to validate the internal syntax of MAGE-ML documents in the Public Workspace.

Oncomine

Oncomine (www.oncomine.org) is a cancer-specific gene expression data resource. It collects and analyzes published cancer gene expression data. Unlike the resources described previously, it does not allow submission or download of data sets. Instead, it provides expression values of genes across thousands of cancer samples or

permits the exploration of genes, processes, and pathways in various types of cancer.

Mitelman Database of Chromosome Aberrations in Cancer

The information in the Mitelman Database of Chromosome Aberrations in Cancer (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>) provides data that have been manually culled from the literature that relate chromosomal aberrations to tumor characteristics. The site contains information on more than 50,000 cases and is updated quarterly. Served through the CGAP Web site, a collection of tools facilitate data access. A **Cases Quick Searcher** allows query of individual patient cases using the four major fields: aberration, breakpoint, morphology, and topography. The **Cases Full Searcher** permits a more detailed search of the same individual patient cases by including more cytogenetic field choices and adding search fields for patient characteristics and references.

The Mitelman resource has two feature of significance to molecular etiology. The **Molecular Biology and Clinical (MBC) Associations Searcher** searches curated studies pertaining to gene rearrangements and clinical associations of cytogenetic aberrations. The **Recurrent Chromosome Aberrations Searcher** provides a way to search for structural and numerical abnormalities that are recurrent (i.e., present in two or more cases with the same morphology and topography).

Pathway Interaction Database

The Pathway Interaction Database (PID; <http://pid.nci.nih.gov>) is a resource of network-level representations of interactions and pathways (Figure 24-4). The PID contains data from two sources. The most robust data originate from curation of pathway information by Nature Publishing Group under contract to the National Cancer Institute. Labeled in PID as the "NCI–Nature Curated" data are annotated with citations and evidence codes. It captures relevant post-translational modifications of proteins. Each pathway is verified by one or more experts in the field. All data in the database are from humans. An older source consists of manually encoded pathways and interactions extracted from the database of pathways posted on the BioCarta Web site (<http://www.biocarta.com>) prior to June 2004. A Web query interface supports browsing across predefined pathways, construction of larger networks around molecules and predefined pathways of interest, and the analysis and visualization of lists of targeted molecules in the context of predefined and novel networks.

Analytic Tools

There is a large armamentarium of bioinformatics applications. There are excellent software suites maintained by commercial firms. These commercial tools may represent the reason the business exists, such as Rosetta Biosoftware (<http://rosettahbio.com>), bioinformatics extensions of other analytic software, such as SAS (www.sas.com) or MATLAB (www.matlab.com), tools to expand other lines of business that support molecular biology, such as

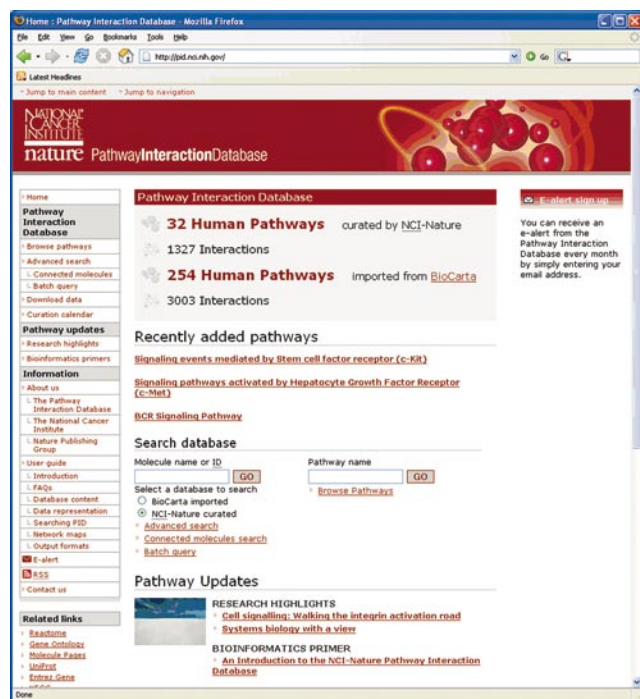


FIGURE 24-4 PATHWAY INTERACTION DATABASE HOMEPAGE. Shown here is the World Wide Web portal that permits access to curated information on canonical biologic networks and pathways.

Stratgene (www.stratgene.com) or additional functionality added to existing software applications, such as Oracle Corporation (www.Oracle.com). Commercial software offers many advantages to user. These include professional support, reliable bug fixes, and commercial documentation and user guides. They also are expensive and closed (not open source), the latter representing potential barriers in implementation and extension.

Complementing commercial software is an expanding collection of open-source software, largely developed and supported by the academic community. The strength of this software is its diversity, availability, and customizability. It also varies dramatically in quality, robustness, and sophistication.

Tools for Bioinformaticians

A library of open-source software is maintained by the **Open Bioinformatics Foundation** (www.open-bio.org) a nonprofit, volunteer organization. This effort grew out of programming language-specific application development efforts including Bioperl, BioJava, and Biopython (see following paragraphs). The foundation does not develop software, it provides the administrative organization necessary to create an electronic clearinghouse of resources. Although it is difficult to generalize such a diverse resource, the library of materials is geared toward bioinformatics developers or individuals with knowledge of computer programming. Although applications can be user-friendly, use of the resource is not intended for individuals without some computer sophistication. Nevertheless, it is a tremendous resource for bioinformatics experts. Programming language-specific components are discussed in the following sections.

Bioconductor

Bioconductor (www.bioconductor.org) is an R programming language open-source and open-development software project for the analysis and interpretation of genomic data. Open development means that the community is made aware of the development plans for each of the tools and in some instances, encouraged to contribute additions and modifications to the software itself. A primary focus of the effort is microarray data analysis, although many of the software tools are general and can be used for the analysis of DNA sequence or single-nucleotide polymorphism (SNP) data. There are two releases of Bioconductor every year.

BioPerl

BioPerl (BioPerl.org) is a Perl programming language open-source and open-development software toolkit to support all aspects of bioinformatics. The toolkit is divided into several packages, all of which extend and require the use of the **Core package**. A **Run package** provides wrappers for executing more than 60 common bioinformatics applications.

There are specific packages that support the manipulation of genotype and phenotype data for linkages studies (the **Pedigree** package), manipulating microarray data formats (the **Microarray** package), analysis of protein-protein interaction data (the **Network** package), and a database to store sequence and annotation data (**BioPerl db**). For developers, there are packages that support the creation of analytic pipelines (the **Pipeline** package), basic widgets in Perl-Tk to support GUI development (the **GUI** package), and C programming language extensions to support sequence alignment and an interface to the Staden IO library (the **EXT** package).

BioJava

BioJava (BioJava.org) is an open-source and open-development Java programming language software toolkit to support the processing of biological data. It includes objects for manipulating biologic sequences, file parsers, distributed annotation service (DAS) client and server support, access to *Ensembl* databases, tools for making sequence analysis GUIs, and statistical routines including a dynamic programming toolkit.

Tools for End Users

Many applications have been developed to support laboratory researchers without programming or biostatistics background ("end users"). Although requiring computer technical expertise to set up, they are designed to support analysis of multiple types of data. Tools highlighted here have been developed to work together using the cancer Biomedical Informatics Grid (caBIG) framework. A complete inventory of more than 25 caBIG-compatible bioinformatics tools is available at <http://caBIG.nci.nih.gov/inventory>. In addition to providing download capability for the tool, the inventory also provides summaries of the individual applications, demonstration, and exercise material related to the tool and end-user support resources (Figure 24-5).

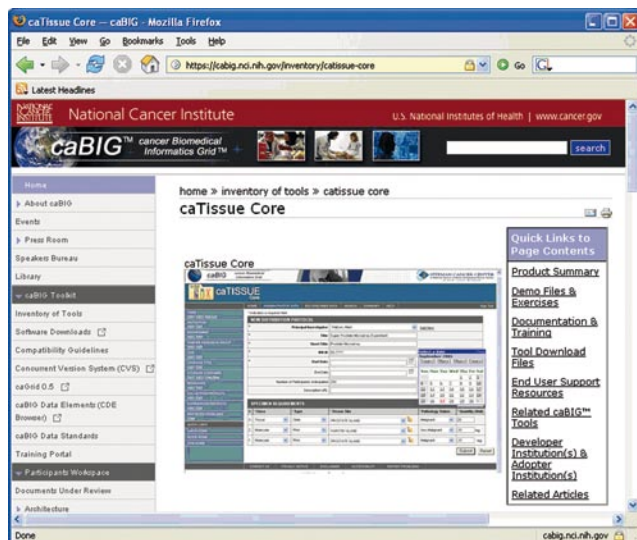


FIGURE 24-5 A CANCER BIOMEDICAL INFORMATICS GRID (caBIG) TOOL INVENTORY PAGE. Shown here is a sample World Wide Web page describing a caBIG-compliant software application, caTissue Core. Each such page provides a product summary, demonstration files and exercises on how to use the application, as well as other caBIG tools that interoperate with or compliment the functionality of the described tool.

geWorkbench

geWorkbench (genomics Workbench <http://wiki.c2b2.columbia.edu/workbench>) is a Java-based open-source platform that offers a comprehensive and extensible collection of tools for the management, analysis, visualization, and annotation of biomedical data. For microarray data it provides components for filtering and normalization, basic statistical analyses, clustering, network reverse engineering, and data visualization. For sequence data it provides routines such as BLAST, pattern detection, transcription factor mapping, and syntenic region analysis. geWorkbench's key feature is its integration. It allows a user to move from one data type to another in a seamless fashion. For example, genomic sequences around genes of interest found in microarray experiments can be retrieved and used for promoter/transcription factor analysis. The geWorkbench platform can use caBIG's caGrid services.

Developed by bioinformaticists at Columbia University, geWorkbench represents a strategy in which end-user tools are tightly coupled within a single application. This tight coupling provides seamless transition among the various components required to perform bioinformatics analysis of data. However, it is also rigid in that users cannot add analytic or processing steps not anticipated by the development team.

GenePattern

GenePattern (www.broad.mit.edu/cancer/software/genepattern/) is an analysis workflow tool that supports multidisciplinary genomic research. Users create analysis pipelines that chain together various analytic steps required to perform a desired analysis. More specifically, the output of one step can be used as the input of another. These pipelines are constructed through a common application interface that allows users to gain access to a repository of

analytic tools for various data types. An interesting capability of GenePattern is its ability to capture, automate, and share the steps used to perform the analysis. More specifically, its workflow captures the computational analysis methods and their parameters. Once created, the pipeline can reproduce the steps used in an analysis or be reapplied to novel data sets. GenePattern's pipelines can be exported and imported, allowing them to be shared or used to document published research.

GenePattern supports class prediction, class discovery, pathway analysis, and marker selection gene expression analyses. It also supports analysis of high-density SNP data for the determination of copy number alterations (amplifications and deletions), and loss of heterozygosity detection.

For bioinformaticians, GenePattern provides APIs that permit the use of analysis modules developed in Java, MATLAB, and R (see preceding sections). GenePattern uses caBIG's caGrid services. This means GenePattern can use data available through caBIG's federation and the GenePattern can be set-up as an analytic service.

Developed by bioinformaticists at the Broad Institute, GenePattern loosely couples its analytic pipeline. This loose coupling provides flexibility in designing analytic pipelines and the capacity to add new routines or processing steps as they are identified by the community. However, this flexibility comes with the end-user price of requiring more knowledge of the inputs and outputs of various modules.

WebGenome

WebGenome (<http://WebGenome.nci.nih.gov>) is an open-source, Java-based application tool suite for plotting and visualizing microarray-based gene expression and comparative genome hybridization data. It is an end-user-oriented application that enables its users to create and manage virtual experiments and analytic pipelines. It is visually oriented and supports various plots of data, including annotation plots showing the location of annotated genome features in relation to measured DNA copy number, regions of loss and gain in relation to chromosome ideograms, and plots of specific reporter probes.

WebGenome works with caBIG's caGrid services. This means WebGenome can use data available through caBIG's federation and the WebGenome can be set up as an analytic service.

Biomedical Data Integration

As indicated in the preceding sections, biomedical data is voluminous and diverse. It may also be fragmented in location. This fragmentation may be due to storage in separate institutional databases designed to meet specific needs. Alternatively, it may be geographically distributed such as in a federation such as caBIG or more loosely distributed across multiple laboratory Web sites. To efficiently manipulate the data, it needs to be integrated and aggregated. This is effectively done through the creation of data marts. A data mart (or data warehouse) is a specialized type of database that combines and integrates data in a manner that facilitates its retrieval and use in analysis.

caIntegrator

caIntegrator (<http://caIntegrator.nci.nih.gov>) is an open-source data mart that provides a mechanism for integrating and aggregating biomedical research data (as shown in Figure 24-1) and provides access to a variety of data types (e.g., microarray-based gene expression, immunohistochemistry, SNP, clinical trials data, etc.) in a cohesive fashion. The caIntegrator platform interfaces with caBIG-compatible databases, such as caArray, and creates a stand-alone data warehouse. Depending on the particular user's role, he or she is permitted access to specific sets of the study data. A series of tools enables the user to easily analyze and interact with the integrated data to achieve greater insight into molecular characteristic of tumors and correlate these characteristics with clinical outcome.

caIntegrator uses a basic "star schema" as its database structure with modification for the study data warehouse design that supports the integration of clinical and genomic data. It is a generic, query optimized schema that contains tables such as "Differential_Gene_Expression_Fact" and "Genomic_Abnormality_Fact." Look-up entities such as Genes, Biosample, and Disease type make up the dimensions in the schema. This schema provides a highly denormalized view of the data (duplicate representation of data) and a data neutral framework from which queries can be executed with quick retrieval time.

The overall goal of the caIntegrator project is to provide a framework with the infrastructural components needed to develop enterprise level translational applications. One such reference implementation is **REMBRANDT** (Repository of Molecular BRAin Neoplasia DaTa), <http://rembrandt.nci.nih.gov>. REMBRANDT is a powerful and intuitive informatics system designed to integrate genetic and clinical information from brain tumor clinical trials for improved research, disease diagnosis, and treatment. It provides researchers with the ability to perform ad hoc querying and reporting across multiple data domains, such as gene expression, chromosomal aberrations, and clinical data. Scientists are able to answer basic questions related to a patient or patient population and view the integrated data sets in a variety of contexts. Tools that link data to other annotations such as cellular pathways, gene ontology terms, and genomic information are embedded within this system. Figures 24-6 through 24-8 illustrate the key features in REMBRANDT implemented using the caIntegrator framework.

Additional implementations include the **I-SPY** (Investigation of Serial Studies to Predict Your Therapeutic Response with Imaging and Molecular Analysis) breast cancer trial **CGEMS** (Cancer Genetic Markers of Susceptibility; <http://CGEMS.cnacn.gov>) and The Cancer Genome Atlas (**TCGA** <http://cancergenome.nih.gov>) data portals. The primary objective of the I SPY Trial is to identify surrogate markers of response to neoadjuvant chemotherapy that are predictive of pathologic remissions and survival in stage 3 breast cancer. The goal is to identify molecular markers and/or magnetic resonance imaging results that predict 3-year disease-free survival in these patients. CGEMS is an NCI project to identify genetic alterations that make people susceptible to prostate and breast cancers. The goal of CGEMS is to analyze the entire genome for most common types of SNPs that are associated with each of these diseases. TCGA is a comprehensive and

KAPLAN-MEIER SURVIVAL PLOT FOR SAMPLES WITH DIFFERENTIAL EGFR GENE EXPRESSION

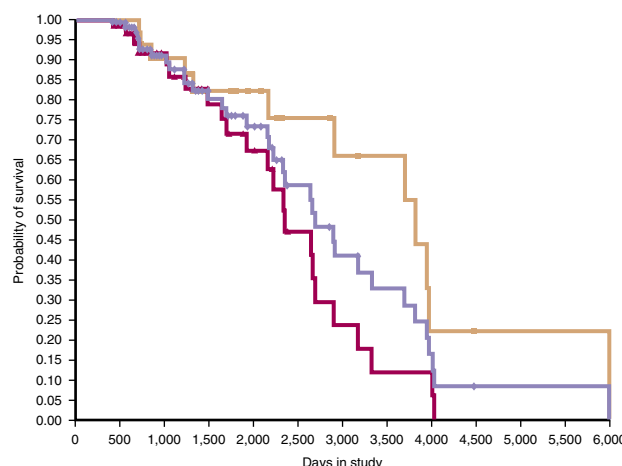


FIGURE 24-6 Kaplan-Meier survival plot based on gene expression data integrate gene expression data with clinical outcome generated from REMBRANDT Web portal. Users can query gene expression and graph changes in survival rate at each time point on the study. Kaplan-Meier (K-M) estimates are calculated based on the last follow-up time and the censor status (o, alive; 1, dead) from the samples of interest. The Kaplan-Meier estimates are then plotted against the survival time. Users can dynamically modify the fold change (up- and down-regulation) thresholds and redraw the plot. A log-rank p value is provided as an indication of significance of the difference in survival between any two groups of samples segregated on the basis of gene expression of the gene of interest.

coordinated effort to accelerate our understanding of the molecular basis of cancer through the application of genome analysis technologies, including large-scale genome sequencing.

The caIntegrator framework contains a common set of interfaces (APIs) and specification objects that define clinical genomic analysis services. For statistical analysis it uses R (see preceding section). The Rserve package (<http://stats.math.uni-augsburg.de/Rserve/>) is used to interface the R system with Java. Rserve provides Java classes to execute R commands and to retrieve results

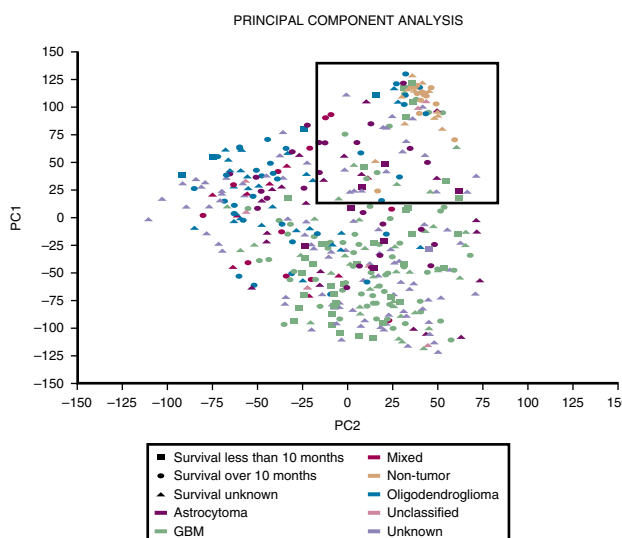


FIGURE 24-7 This figure shows an example principle component analysis (PCA) report from the REMBRANDT application. This two-dimensional graph plots the various principal components from the gene expression PCA analysis. Various analysis options are provided to the user to select from an array of gene/reporter filtering and sample selection settings.

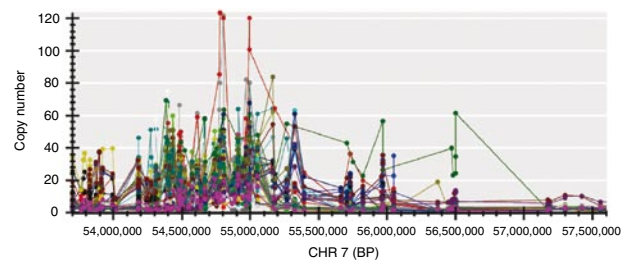


FIGURE 24-8 Scatter plots from the REMBRANDT Web-portal display measured copy number against physical genome location in an application called webGenome, which has been seamlessly integrated with calIntegrator. These plots are context-sensitive to the copy number reports generated from the copy number queries in the calIntegrator application. You can view data at arbitrary resolutions from the entire genome on down.

as Java objects. The overall architecture of the Analytical Server allows the user to plug in any other statistical package such as SAS (<http://www.sas.com>) that exposes an API.

Architecture, Infrastructure, and Virtual Cancer Research Laboratories

Traditional approaches in bioinformatics necessitate scientists establishing IT capabilities local to their laboratory, organization, or institution. This requires groups to download and install a myriad of data and programs. The bioinformatics community supports this through the ethos of open source and open access to its data and tools. Nevertheless, this approach represents a tremendous

burden of creating/recreating infrastructure. For example, creating a copy of the UCSC Genome Browser and its data requires significantly more than a terabyte of disk storage. Although hardware is increasingly not a rate-limiting step in using IT, the complexity of installation still requires computer professionals.

An alternative is to leverage the power of the Internet. Interestingly, a key factor in the creation of the Internet was to facilitate scientists working in the United State’s national laboratories to share data and computer infrastructure. Similarly, the World Wide Web represents an Internet extension that was created within the National Supercomputer Facilities to facilitate the sharing of content.

As implied above, the Internet and Web are widely used within the biomedical research community to exchange data, applications, and literature. The search engine as discovery and integration infrastructure has been essential to the ongoing success of this approach. However, more technologically sophisticated alternative approaches to the use of the Internet have been slow to penetrate biomedicine. These approaches have transformed the finance and business communities (as well as the military) and represent the substance of e-business.

This next generation use of the Web uses Web-based-services where data is electronically transmitted in structured fashion interpretable by computers and applications share their capabilities by receiving this electronic information without having to be installed locally. Agreement to use common standards and API definitions generates **semantic interoperability**. With semantic interoperability, diverse applications can use multiple, distributed sources of data and share the output of their analysis with other applications. Infrastructure, applications, and data resources

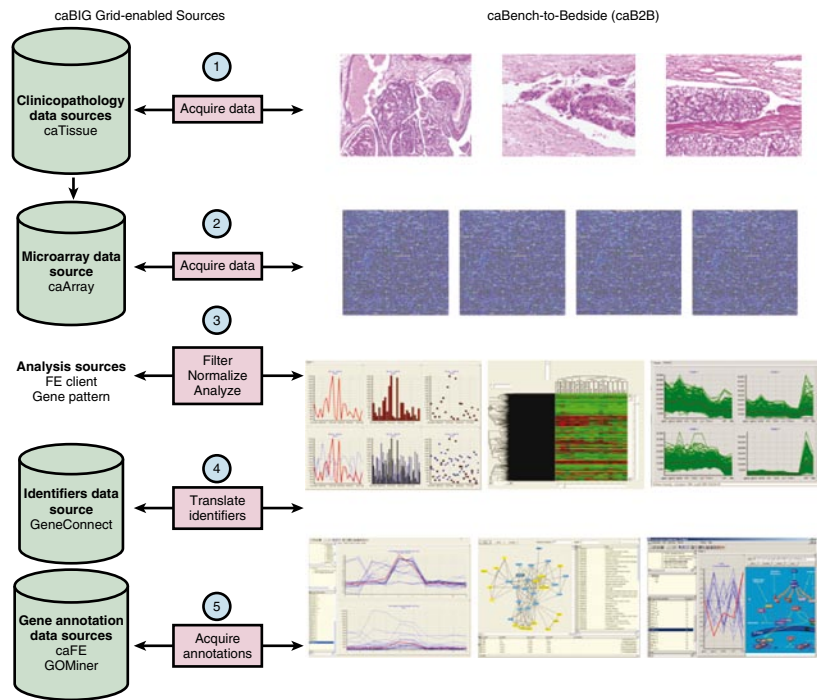


FIGURE 24-9 ILLUSTRATION OF THE CAGRID-BASED TOOL, CABENCH-TO-BEDSIDE (CAB2B). Shown here the variety of caGrid services that caB2B navigates and integrates to support translational research activities.

that adhere to a common set of standards constitute a **services-oriented architecture (SOA)**.

The cancer community has created such a next-generation World Wide Web, the Cancer Biomedical Informatics Grid (**caBIG**). CaBIG is a voluntary network or grid connecting individuals and institutions to enable the sharing of data and tools. The goal is to speed the understanding of the etiology of cancer and subsequent delivery of innovative approaches for prevention and treatment. Members connect resources using the open-source, SOA infrastructure, **caGrid** (<http://gforge.nci.nih.gov/projects/cagrid-1-0>).

Installation and use of caGrid SOA “middleware” infrastructure (specialized software that connect data and applications) requires bioinformatics technical expertise. However, applications built on top of this infrastructure can be end-user oriented and abstract the complexity of using caGrid in a manner similar to how Web browsers hide the technical complexity of the Internet and permit access to highly sophisticated applications (e.g., Google search engine application). Two examples of applications that use the caBIG network to create a virtual bioinformatics platform are summarized in the following paragraphs.

The first, the cancer Translational Research Informatics Platform, **caTRIP** is a framework developed by Duke University to

query and mine clinical, pathology, gene expression, and proteomics data. It uses the caGrid infrastructure to join numerous caBIG tools and data within the borders of a single institution firewall for security. However, its use of caGrid permits previously separate organizations (the Duke Breast Oncology SPORE and Cancer and Leukemia Group B NCI Cooperative Group) to use a common informatics infrastructure.

The true promise of the caBIG virtual infrastructure is realized in the Washington University of St. Louis’s application: cancer Bench to Bedside (**caB2B**). Like business-to-business applications supporting e-commerce, caB2B uses components drawn from the nationwide caBIG partners ([Figure 24-9](#)). These include the caArray data resource and the GenePattern analytic engine described in the preceding sections. Additional caBIG services (caTissue, a grid-enabled tissue banking system; GeneConnect, a genomic identifier mapping service; Function Express [caFE], an automated microarray annotation system; and GOMiner, Gene Ontology annotation system) are also drawn upon by the application. The caB2B application manages the flow of work necessary to make basic scientific discoveries and translate these into clinical research settings. It leverages the distributed nature of caBIG, permitting the use of data and services distributed nationwide.

Early Detection of Cancer

Molecular Screening

Important progress has been made in the early detection of certain forms of cancer. Newer radiologic techniques such as ultrasound and magnetic resonance imaging (MRI) afford increasingly more sensitive noninvasive diagnostic procedures for detection of small tumors. However, although patients with certain tumor types have benefited from such radiologic advances, patients with other cancers such as epithelial ovarian and pancreatic tumors have not. These and other tumor types continue to be commonly diagnosed at an advanced stage and are thus associated with poor patient outcomes. Further, as radiologic and other techniques that facilitate early detection have primarily provided conventional tumor staging information (e.g., tumor size and lymph node status), they do not facilitate individualization of treatment decision making because cancer is a markedly molecularly heterogeneous disease. Thus, pathophysiologic characterization of cancer using molecular biomarkers is likely the key to advancing early diagnosis and treatment. A biomarker is any characteristic that can be evaluated as a measure of normal biologic or pathogenic processes or of the potential efficacy of a specific therapeutic intervention, and thus may be any component of the tumor genomic, proteomic, carbohydrate, or lipid makeup or any biophysical characteristic of tumor cells or tissues. Because biomarkers can be derived from many different approaches and have multiple potential uses, it is important to designate these as biomarkers for a particular function (e.g., biomarkers for early diagnosis, prognostic biomarkers, or pharmacodynamic biomarkers). Novel molecular screening techniques have the potential to address tumor molecular heterogeneity, revolutionize early cancer detection and at the same time facilitate accurate risk assessment and individual treatment planning. This chapter explores clinical practice and the discovery and validation of novel molecular biomarkers for early detection of cancer. Since early detection and prevention are in some regards a continuum as they relate to the issue of high risk, cancer prevention will also be addressed (1–4).

Screening for Early Detection

Early detection implies the diagnosis of cancer at an early stage in its development and is thus generally expected to result in improvements in patient outcomes using conventional treatment strategies. Screening is a term often used for approaches that facilitate early cancer detection. Screening technologies must be capable of detecting small tumors at an early stage. Importantly, they should identify tumors at a stage when they can be cured by surgery alone or when they are more responsive to therapy, thus improving patient mortality and morbidity. Thus, screening has the potential to improve patient outcomes, as shown in the case of epithelial ovarian cancer in [Figure 25-1](#). Further, an effective cancer screening approach must be cost-effective, acceptable to patients, and associated with limited morbidity ([Figure 25-2](#)). Since screening of the entire population is often not practicable, guidelines for patient risk assessment are also often necessary to appropriately target technologies for the purposes of prevention and early cancer diagnosis. This allows application of screening tests to those most likely to benefit from the approach and also alleviates some of the concerns about challenges associated with false-positive results. As our understanding of the molecular heterogeneity of cancer rapidly advances, novel criteria can be added to these and other conventional criteria (e.g., sensitivity and specificity) that describe an ideal screening biomarker. Thus, as most cancer treatments are effective in only a minority of early-stage cancer patients, useful screening biomarkers will also facilitate assessment of likely outcome (risk assessment) and guide appropriate therapies in individual patients.

A Good Screening Test Versus a Suitable Cancer for Screening

Although it obviously has intuitive appeal, earlier diagnosis is not necessarily better or worth the cost. There are four critical terms

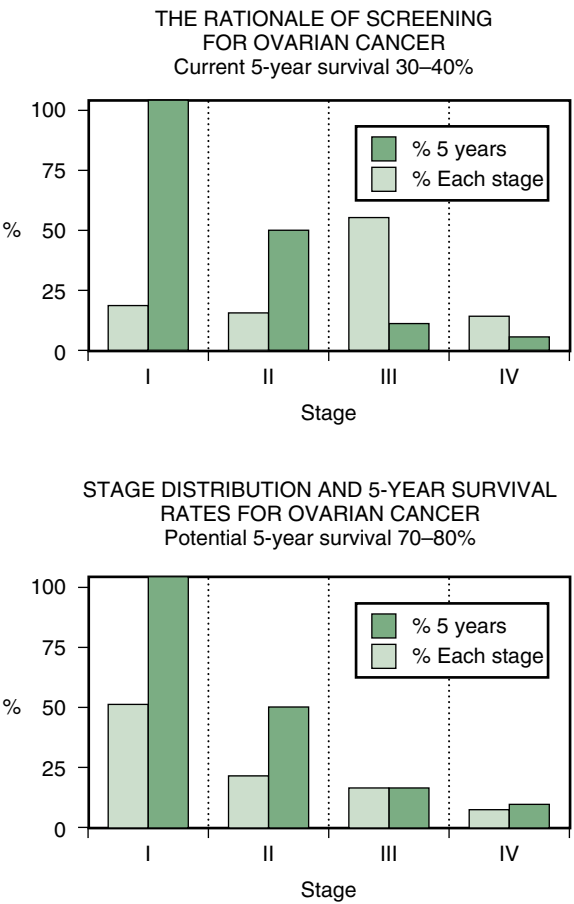


FIGURE 25-1 THE POTENTIAL IMPACT OF SCREENING ON CANCER MORTALITY. Currently, most cases of ovarian cancers are advanced (stage III) at diagnosis. However, effective screening has the potential to dramatically improve 5-year survival (5YS) by allowing diagnosis of most cases at an early stage (Jacobs).

that describe the validity of a screening test: sensitivity, specificity, and positive and negative predictive values. Sensitivity, one measure of how good a test is, is the proportion of cases of the disease that are detected by the test (number of true positives/number of true positives + false negatives). Specificity is the proportion of cases without disease that are detected as negative by the test (number of true negatives/number of true negatives + false positives). When a test result is a continuous variable, sensitivity and specificity are inversely related and a cutoff for the presence of disease will be placed in an arbitrary fashion that generally indicates the clinical effect of a wrong result (Figure 25-3). In some cases, the sequencing of two tests for screening (a primary and a secondary test) can allow optimization of sensitivity and/or specificity beyond that achievable with only the primary test prior to definitive treatment (e.g., with ultrasound-guided breast biopsy following mammography but before definitive breast surgery). The prevalence of the disease in a population also affects screening test performance: In low-prevalence settings, even very good tests have poor positive predictive values (the proportion of patients with positive test results who are correctly diagnosed [i.e., number of true positives/number of true positives + false positives]). Hence, knowledge of the approximate prevalence of the disease is a critical prerequisite to interpreting screening test results. It is thus easier to effectively screen for common diseases, an inherent problem with devising effective screening strategies for diseases such as ovarian and pancreatic cancers.

There are also disease-specific considerations for successful implementation of screening. Lead time and length biases are important considerations (Figure 25-4); these biases can distort the apparent value of screening programs, and randomized controlled trials are the only way to avoid them. Furthermore, the presence of a premalignant stage likely facilitates and may be important to the development of an effective screening strategy. For utility, a screening test must demonstrate a high degree of selectivity to a particular tumor type. It could be challenging to have a test that

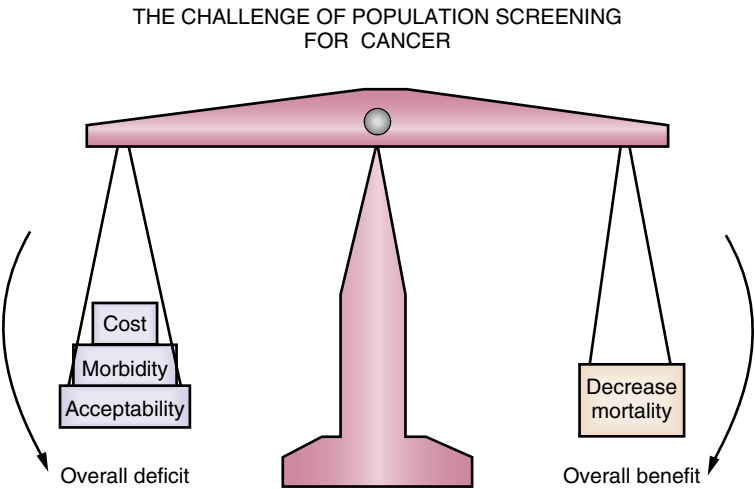


FIGURE 25-2 The utility of a cancer screening approach will depend on a balance between its impact on patient outcomes and its cost, associated side effects (morbidity), and acceptability to patients (Jacobs).

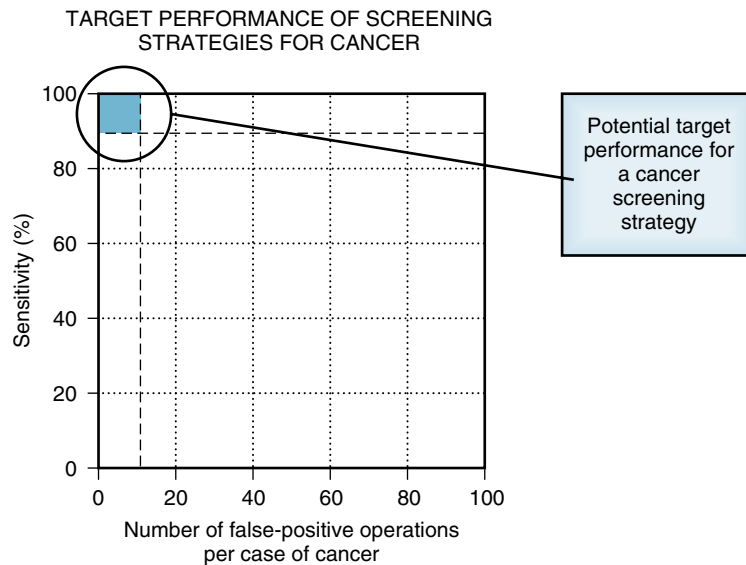


FIGURE 25-3 Biopsies and surgery are definitive ways of diagnosing and treating many forms of cancer. Thus, a useful screening test must have high specificity and sensitivity. In the absence of a high specificity, an unacceptably high proportion of patients will be subjected to an unnecessary biopsy and/or surgery (Jacobs).

indicates that a patient has an increased risk of carrying a cancer, without knowledge of which type of cancer. This is particularly important for uncommon diseases such as ovarian and pancreatic cancers. Because the inappropriate application or interpretation of screening tests can raise needless anxiety, initiate unnecessary and harmful diagnostic testing, and squander health-care resources, these concepts are crucial considerations for the implementation of a successful screening strategy.

Clinical Practice

The major factors that are used to guide and/or effect cancer screening are risk determination, regular self assessment and/or physician examination, strategic use of radiologic imaging approaches, and use of a limited number of molecular markers.

Risk assessment is initially used to stratify a patient to screening or, if the risk is high enough, prevention or prophylactic surgery. It is largely based on patient-specific factors that are determined from a routine history and physical examination, including age, family history, where appropriate genetic testing, and social factors such as tobacco use. Age is one of the most important cancer risk factors, as is family history. Family history is a key factor in identifying patients who may benefit from genetic testing to establish a more accurate risk prediction. Risk prediction also involves a limited number of specific molecular biomarkers. For example, serum prostate-specific antigen (PSA) measurement is now routinely recommended in men older than 50 years because of the frequency of prostate cancer in this age group, although PSA screening remains controversial and not routinely recommended in the absence of definitive proof that it decreases prostate cancer death rates (Box 25-1; 5).

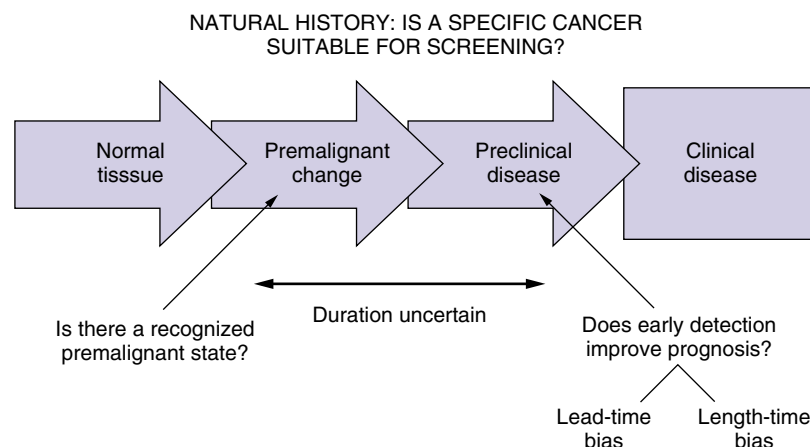


FIGURE 25-4 DISEASE-SPECIFIC CONSIDERATIONS FOR IMPLEMENTATION OF SUCCESSFUL SCREENING. The presence of a recognized premalignant stage facilitates effective screening. Lead-time bias occurs when early diagnosis falsely appears to prolong survival. Length-time bias occurs when screening overrepresents less aggressive disease (e.g., with prostate cancer) (Jacobs).

Box 25-1

Prostate-specific antigen (PSA) is normally present in the blood at very low levels; normal PSA levels are between 0 and 4.0 ng/mL. Increased levels of PSA may suggest the presence of prostate cancer. However, prostate cancer can also be present in the complete absence of an elevated PSA level. PSA levels can be also elevated due to prostate infection, irritation, benign prostatic hypertrophy (enlargement) or hyperplasia (BPH), or recent ejaculation. Thus, PSA is not an adequately sensitive or specific marker for prostate cancer. However, the PSA rate of rise may have value in predicting prostate cancer prognosis. Men with prostate cancer whose PSA level increased by more than 2.0 ng/mL during the year before the diagnosis of prostate cancer had a higher risk of death from prostate cancer after radical prostatectomy.

Most PSA in the blood is bound to serum proteins. A small amount is not protein bound and is called free PSA. In prostate cancer the ratio of free PSA to total PSA is decreased. The risk of cancer increases if the free to total ratio is less than 25%. Measuring the ratio of free to total PSA may be particularly promising for eliminating unnecessary biopsies in men with PSA levels between 4 and 10 ng/mL.

In families with a significant history of breast and ovarian cancers, key cancer risk (usually hereditary) biomarkers can now be identified in many cases. Clinical models can predict an individual woman's probability of having a genetic mutation based on the family history (e.g., BRCAPRO model; 6). The incorporation of such models into assessment of a woman with a significant family history of breast or ovarian cancer can be useful in guiding specific molecular tests that further stratify risk and guide subsequent management (screening or prevention). *BRCA1* and *BRCA2* are well-known inherited predictive biomarkers of high breast and ovarian cancer risk (7), and mutations in these genes account for 5% to 10% of all breast cancers and for 65% or more of inherited breast cancers (8,9). *BRCA1/BRCA2* mutations are associated with a 55% to 87% lifetime risk of developing breast cancer, in addition to a mutation type-specific high risk of ovarian cancer (10). In addition, approximately 12% of high-risk women found to be negative for *BRCA1* and *BRCA2* mutations are estimated to carry another cancer-predisposing genomic alteration (11). This suggests that improved testing approaches and identification of additional biomarkers of risk are needed. Less-well-studied breast cancer susceptibility genes include *CHEK2*, *ATM*, *NBS1*, *LKB1*, *PTEN*, *p53*, *XRCC1*, and *STK11* but these are not commonly sequenced due to the rarity of inherited mutations in breast cancer patients (12–14).

Key familial cancer risk biomarkers have also been identified for colorectal cancer (15). Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colon cancer (HNPCC) represent predisposing genetic syndromes for early-onset familial/hereditary colon cancer. These syndromes are characterized by germ-line mutations in the adenomatous polyposis coli (APC) and deoxyribonucleic acid (DNA) mismatch repair genes, respectively. Management guidelines entail cancer prevention with FAP (i.e., colectomy) and intensive screening with HNPCC (because of the lower risk of colorectal cancer associated with the latter). In addition, persistent inflammatory bowel disease (chronic ulcerative or Crohns colitis) place patients at high risk for colorectal cancer.

Regular self assessment and physician examination are important in screening for early cancer detection. Greater

population awareness increases the effectiveness of such strategies. Specific screening recommendations that involve diagnostic procedures are generally only applied to the population when there is conclusive evidence of an associated survival benefit and when the cancer is a common cause of mortality, the latter for reasons that include cost effectiveness. Thus, to facilitate earlier detection of colorectal cancer, and with compelling evidence that removal of adenomas can prevent incident cancers, it is now recommended that people be screened beginning at 50 years of age by annual fecal occult blood testing and/or one of the following: flexible sigmoidoscopy or double-contrast barium enema every 5 years or colonoscopy every 10 years (16,17). Increasingly, the ability of colonoscopy to visualize small lesions throughout the entire colon has made this the screening method of choice.

Many efforts that facilitate early detection of cancer rely on radiology. For example, annual mammography is recommended for women aged 40 years and older in the United States, although the applicability to women younger than 50 years remains somewhat controversial. Multiple statistical models have shown that mammographic screening has helped reduce the rate of death from breast cancer in the United States (18). Mammographic screening has only limited sensitivity and specificity, particularly in premenopausal women. However, as it is part of a two-step approach, with mammography indicating in most cases an increased risk, followed by a definitive approach of needle biopsy, it is an effective approach. A similar two-stage approach allows the relatively low specificity of "Pap" smears combined with the high specificity of colposcopy and biopsy to provide an effective approach. Studies are also investigating imaging modalities as initial screening for other forms of cancer. Although routine chest radiography has not been shown to improve outcomes of lung cancer patients, computed tomography (CT) is much more sensitive and is the subject of current lung cancer screening studies (e.g., NELSON trial) in people at increased risk from cigarette smoking (19). These studies remain controversial, particularly as several nonrandomized studies have suggested efficacy. Improved imaging techniques have the potential to contribute dramatically to our ability to detect cancer at an early stage. Radiologic imaging is usually also necessary for definitive documentation of early tumors that are detected by other screening approaches before biopsy confirmation (e.g., with an elevated PSA). These two-stage approaches can increase test specificity sufficiently to allow use for relatively low-prevalence cancers. Indeed, for low-prevalence diseases, a high specificity remains the dominant required characteristic, whether achieved using a single- or two-stage approach. Further, investigative molecular approaches to facilitate early diagnosis of cancer are using novel imaging techniques, as will be discussed briefly later in this chapter (20,21).

More specific guidelines have been developed for high-risk patients with strong family histories of cancer, particularly for carriers of genomic mutation biomarkers that are associated with a very high risk of cancer. When the cancer risk associated with the specific (usually hereditary) biomarker is high enough, recommendations focus on prevention rather than early detection. Since prevention strategies generally impact quality of life to a greater degree than does screening, the identification of inherited mutations associated with a very high cancer risk necessitates patient education and careful,

shared decision making. Dietary and other lifestyle modifications (e.g., exercise) and increased surveillance may be sufficient for cancer prevention in some cases. Prophylactic surgery and chemoprevention are particularly effective risk reduction techniques that are reserved for people at highest risk. Indeed, there is likely a continuum between prevention and treatment of cancer patients, where individuals at particularly high risk may benefit from chemotherapeutic approaches. This “integrative epidemiology” concept may be important for early detection, prognosis, and prediction of response to therapy. Celecoxib is an effective agent for the prevention of colorectal adenomas but, because of potential cardiovascular events, cannot currently be routinely recommended for this indication (22). However, for patients with underlying genetic aberrations, the risk–benefit equation may tilt toward the use of celecoxib and other more toxic agents. Some molecular biomarkers allow risk assessment and facilitate individualized treatment planning. Thus, in breast cancer, the choice of chemopreventive agent should necessitate consideration of the specific mutation. *BRCA2* mutation carriers are at high risk of hormone receptor–positive breast cancer and treatment with tamoxifen or another antihormonal therapy may result in benefit. However, antihormonal therapies have little preventive effect in *BRCA1* mutation carriers since most of these develop hormone receptor–negative tumors. Prophylactic surgery (mastectomy and/or oophorectomy) has been shown to have the greatest protective effect for *BRCA1/2* mutation carriers to date, reducing the risk of breast cancer by up to 90% (7,8,23,24). Many factors (e.g., ethics, cost) must be considered in discussing options for early detection and prevention with very high risk patients and this area of medicine is a rapidly evolving specialty (25–28).

Although approaches to early detection have impacted mortality from certain forms of cancer, there are many tumor types that have not been detected with these approaches and continue to present with advanced disease. Some such tumors, including pancreatic and ovarian cancers, are associated with production of known serum markers (CA19–9 and CA125 [Box 25-2], respectively) but these markers generally lack the necessary sensitivity and, in particular, the high specificity for effective screening. Thus, these tumor types are associated with poor outcomes. However, it can be expected that increasing understanding of the complex molecular

basis of cancer and availability of novel molecular technologies will unearth multiple new approaches to facilitate early detection and possibly ultimately prevention of many forms of cancer.

Discovery of Novel Molecular Markers for Early Detection

Malignant tumors are usually characterized by multiple molecular perturbations that are responsible for the specific behavior of each individual tumor; some of these changes are likely to be detectable from early stages of tumor development. For example, the detection of elevated PSA levels in the serum of men as a result of aberrant production can lead to early diagnosis of prostate cancer. Hence the rationale that molecular technologies may allow identification of sensitive and specific screening biomarkers that will facilitate not only early detection but ultimately also therapy planning (i.e., personalized treatment). Novel high-throughput molecular technologies that comprehensively characterize the cancer genome and proteome are presently adding many new possibilities for identification of screening biomarkers to the more fully explored potential of more traditional and moderate-throughput investigative approaches. Ideally, screening molecular biomarkers will also facilitate prognostication and individualized prediction/treatment planning. In other words, they should themselves be targetable or associated with exploitable molecular targets.

Although the common approach has been to search *de novo* for novel molecular screening biomarkers, another logical approach, and one that should be studied in parallel, is to investigate the potential role in screening of predictive biomarkers that have previously been validated to facilitate individualization of cancer treatment. The latter biomarkers have the advantage of identifying *a priori* a personalized treatment strategy. Thus, for example, ER, PR, and HER2 are conventional predictive factors that are used to guide management of breast cancer patients but these biomarkers may also have potential screening utility for early breast cancer detection. For example, circulating HER2 extracellular domain is detectable in serum and its role in facilitating early diagnosis of HER2 oncogene-amplified breast cancer is beginning to be explored; at present, it is being more intensively investigated for its role in treatment monitoring. Amplification of the HER2 oncogene has been validated as a predictive biomarker for benefit from the monoclonal antibody trastuzumab and, more recently, other HER2-targeted therapies (e.g., pertuzumab, lapatinib; 29). As novel predictive molecular biomarkers of cancer responsiveness to specific therapies are validated, they should also be investigated for a potential role in screening and may allow refinement of therapy planning (30,31).

Novel high-throughput and comprehensive genomic and proteomic technologies are being widely developed for early cancer detection as well as prognostication, prediction, and target identification. These techniques include gene methylation analysis, gene microarrays with analysis of mRNA and miRNA, and comparative genomic hybridization (CGH, e.g., Affymetrix Inc., CA; Agilent Technologies, CA), mass spectrometry/spectroscopy (MS, e.g., Ciphergen, CA; Sequenom Inc., CA), and bead-based

Box 25-2

CA125, an abbreviation for cancer antigen 125, is a tumor marker that may be elevated in the blood of patients with specific types of cancer. It is a mucinous glycoprotein and a product of the *MUC16* gene. CA125 is best known as a marker for ovarian cancer but may also be elevated in other malignant tumors, including endometrial cancer, fallopian tube cancer, lung cancer, breast cancer, and gastrointestinal cancer, and in a number of relatively benign conditions, such as endometriosis, ovarian disorders, pregnancy, and intra-abdominal inflammatory conditions. Thus, CA125 is not sensitive or specific enough for ovarian cancer screening. For example, 21% of all ovarian cancers do not express CA125. Further, CA125 elevation tends to occur more frequently in advanced stage III/IV ovarian cancer. However, CA125 is useful for following ovarian cancer response to treatment, for predicting ovarian cancer prognosis after treatment, and for detecting a recurrence of ovarian cancer. Studies assessing the applicability of changes in CA125 over time followed by ultrasound to improving morbidity and mortality from ovarian cancer are under way.

analysis methods (e.g., Illumina Inc., CA, Luminex, TX). Many of these technologies are only beginning to be applied to the discovery and evaluation of cancer-related molecular marker panels in various clinical settings (32–34). Gene expression profiles have already been extensively explored in identifying good and poor prognosis subsets of various human tumors (35). DNA methylation changes are also common molecular alterations in cancer cells and methylation analysis to detect early cancer cell DNA methylation aberrations in, for example, blood, feces and urine is currently being investigated. Together, these genomic and proteomic platforms have the advantage of comprehensively probing the genome and proteome and thus of identifying a large number of potential biomarkers for follow-up studies. Further, the incorporation of information to develop meta-signatures reflecting global DNA, RNA, and protein abnormalities may be capable of outperforming data derived from a single technology that examines only one of these platforms (36). For example, proteomic studies can augment genomic panels by providing information on post-translational modifications and on the relative levels and activation of proteins. As such data sets become available, systems for effective data management, integrated analysis of pathways, and “meta-analysis” become critical to the successful development of molecular markers (37–39).

Although current molecular markers for early cancer detection and determination of high risk were discovered through study of limited numbers of genes/proteins, the novel

technologies (e.g., gene microarrays) introduced in the preceding paragraph have already been used successfully to develop multi-marker (e.g., multigene) panels for prediction of specific cancer responses to individual therapies and may offer a particularly accurate means of early cancer detection, risk prediction, and planning specific novel cancer treatment and prevention strategies. In our laboratory, we are developing a high-throughput proteomic technology that allows concurrent analysis of the activity and expression of multiple specific kinases and other proteins (40). This reverse-phase protein lysate array is particularly suited to explore the role of kinase signaling in cancer and the molecular effects of novel agents (e.g., tyrosine kinase inhibitors; TKIs) during treatment and in high-risk tissue during chemoprevention (Figure 25-5).

Molecular markers of high risk for cancer or of the presence of a tumor at an early developmental stage may be present in the germ-line genome or may occur in a somatic or acquired fashion in the genome and/or proteome of a particular tissue at high risk of carcinogenesis or possessing early malignant transformation. In addition, evidence of high cancer risk or of early-stage tumors may frequently be present, either in the form of circulating tumor cells or specific markers (e.g., PSA) or groups (“signatures”) of markers in blood or serum. Thus, studies that attempt to improve early cancer detection generally focus on these sites to find novel potentially useful molecular screening biomarkers.

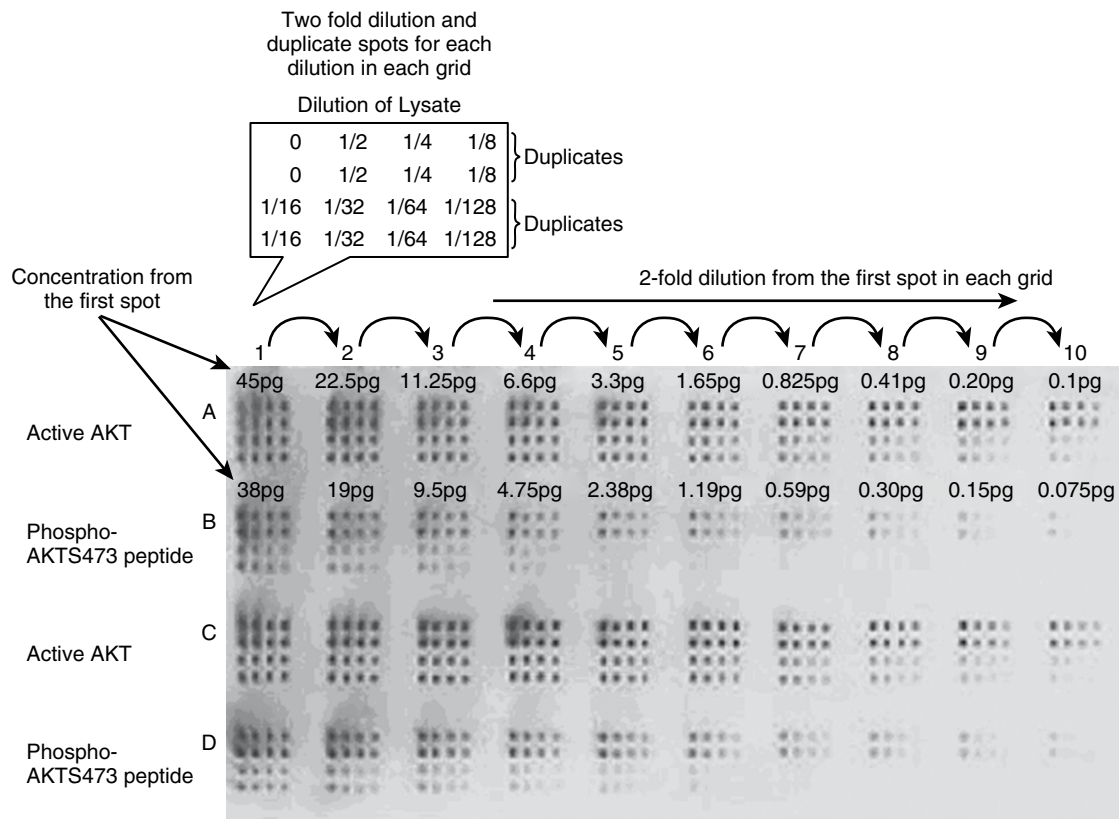


FIGURE 25-5 SENSITIVITY AND REPRODUCIBILITY OF REVERSE PHASE PROTEIN MICROASSAYS. Here, protein lysates from cell lines have been serially diluted on a nitrocellulose-coated slide followed by probing with monospecific antibodies to total or phosphorylated Akt and signal detection and amplification. Serial dilution curves are used for quantification purposes. (From Sheehan KM, Calvert VS, Kay EW, et al. Use of reverse phase protein microarrays and reference standard development for molecular network analysis of metastatic ovarian carcinoma. Mol Cell Proteom 2005;4:346–355, with permission.)

Novel Germ-Line Markers of Risk

Large-scale, whole-genome approaches have been used to uncover novel cancer susceptibility markers (41,42). Kammerer et al. (41) carried out association studies using 16,000 single-nucleotide polymorphisms (SNPs) and identified the intercellular adhesion molecule (ICAM) gene region as a novel breast and prostate cancer susceptibility locus. Similar studies are under way in most prevalent tumor types, with new susceptibility loci being reported frequently. As the ability to accurately predict high risk using novel germ-line biomarkers improves further, early detection and prevention strategies must become increasingly focused on specific molecular subtypes of cancer rather than on the site of cancer per se as the former will be defined by biomarkers of benefit from specific therapies (43).

Tissue-Specific Markers of Early Carcinogenesis and of Carcinogenesis Risk

Although cellular atypia or early malignant change may be present as an early indicator of cancer risk in specific tissues, it is generally extremely difficult to justify the clinical utility of obtaining tissue by biopsy or other means to facilitate early detection of cancer in the absence of methods to adequately stratify high risk. This is because of the morbidity associated with most biopsy procedures, particularly for tissues such as ovary and pancreas that are located deep in the body. However, with availability of less invasive ways to obtain cells likely to be at risk of or to harbor early neoplastic change (e.g., in sputum, bronchial washings, feces, urine, or nipple aspirate), the study of tissue markers for screening of carcinogenesis risk and of early malignant transformation becomes more feasible. Although screening to detect lung cancer at an early stage using routine cytologic examination of sputum did not decrease cancer-specific mortality, the application of molecular detection methods to sputum and bronchial washings is now being studied in an attempt to detect molecular changes associated with premalignant and early malignant bronchial epithelial cells. Fractional allele loss may be a valuable marker for human lung cancer detection in sputum (44). Fluorescent in situ hybridization (FISH) with locus-specific probes to chromosomal regions 5p15, 7p12 (epidermal growth factor receptor [EGFR]), 8q24 (C-MYC), and the centromere of chromosome 6 may also significantly improve the diagnostic sensitivity for malignancy detection in sputum or bronchial brushing and washing specimens (45).

Early malignant cells may be present in feces and advances in fecal immunochemical tests, DNA, and other molecular marker detection methods are being developed in competition with each other as well as with novel endoscopic imaging techniques for the purposes of molecular screening testing for colorectal cancer (46). As with novel approaches under investigation for screening of other tumor types, many of these tests for tissue-specific markers have been developed on the basis of the profile of mutations that are known to occur commonly at different stages of the adenoma–carcinoma sequence in colorectal cancer. Several studies have attempted to detect tumor-specific DNA aberrations including adenomatous polyposis coli (APC) or *Ras* gene mutations or DNA hypermethylation in stool and even plasma to facilitate

early detection of colorectal cancer. In colorectal cancer, several tumor-related genes have been found to have promoter hypermethylation in the CpG islands, and these epigenetic changes are detected in the early phases of colorectal cancer development, even before development of *K-Ras* mutations. MethyLight analysis, a high-throughput quantitative methylation assay that uses fluorescence-based real-time polymerase chain reaction (PCR), of fecal DNA has identified SFRP2 methylation as a sensitive single DNA-based marker for identification of colorectal cancer in stool samples. Whether a combination of genetic and epigenetic markers will identify colorectal cancer at an early stage remains to be shown. Because of the relatively low frequency of any one molecular alteration in colorectal cancer, one-marker assays are more likely to yield high false-negative results. Hence, fecal assays such as the EXACT (EXACT Sciences, Marlborough, MA) multitarget assay panel (47), which targets point mutations at 15 mutational sites on *K-Ras*, *APC*, and *p53* genes, as well as the BAT-26 deletion, a microsatellite instability marker common in proximal colorectal tumors, and high-integrity DNA or “long” DNA (L-DNA), a marker of abnormality in apoptosis, may ultimately prove to be most useful for colorectal cancer screening. In addition to demonstrating efficacy for early diagnosis, an emphasis on enhancing compliance by the introduction of user-friendly tests will clearly be required. Whereas some individuals may opt for colonoscopy as the preferred method of screening, a potential alternative may include the use of fecal or plasma markers as the first of a two-step process wherein only those found to have a high suspicion of early neoplasia will undergo colonoscopy.

Cellular atypia in breast biopsies is associated with a two-to-tenfold increase in the risk of breast cancer. Atypia can also be detected by a number of other less invasive methods including nipple fluid aspiration, ductal lavage, and periareolar fine-needle aspiration (4). The cellular yield from such methods offers the possibility for detailed molecular study of cellular changes associated with atypia and early carcinogenesis, molecular markers leading to a predisposition to cancer in individual women and of potential prevention targets, particularly with the application of novel high-throughput approaches to the study of DNA, RNA, and protein. These approaches are also potentially useful in correlative studies in clinical trials that assess novel chemoprevention agents and are being applied in this setting at M. D. Anderson Cancer Center. Mass-spectrometry (MS)–based approaches can also identify novel biomarkers in ductal lavage specimens and may have a role in the early detection of breast cancer or breast cancer risk (48,49). However, this technology is most applicable to high content analysis of only small numbers of samples.

The cost, invasiveness, lack of large prospective outcome validation studies, and absence of standardized guidelines have confined most of these potentially very useful approaches to small clinical studies.

Serum Markers

Serum markers have clear potential utility in identifying patients with early-stage cancer as well as those at high risk who may benefit from chemoprevention. Urine is another potential source of tumor markers for genitourinary cancer screening (50). Serum

markers also offer a foreseeable means by which to better facilitate treatment planning and individualization of care than is possible. Conventional serum markers include CA19–9, CEA, CA125, CA15–3, CA27.29 (CA27.29), tissue polypeptide antigen (TPA), tissue polypeptide-specific antigen (TPS), and the shed form of HER2 (51,52). Although these biomarkers are used routinely for monitoring the course of disease, their application to screening is limited by their lack of adequate sensitivity and specificity (53). Specificity can be improved by monitoring increases in individual marker levels over time but panels of markers will almost certainly be required to increase sensitivity for use in routine cancer screening. The conventional concept concerning the screening utility of serum biomarkers is that their detection should subsequently trigger clinical assessment by imaging and biopsy, or increased surveillance if appropriate. Alternatively, novel serum markers might be used following screening by other means to increase the specificity of the latter approach. Thus, a novel biomarker may allow definition of an equivocal mammographic lesion as appropriate for serial monitoring or immediate biopsy. It is unlikely that serum markers will have sufficient sensitivity, specificity, and selectivity to be used as “stand-alone” approaches, particularly in low-prevalence cancers such as those of the ovary and pancreas. In these cases, the screening test will most likely demonstrate utility in identifying patients who warrant more invasive approaches such as imaging or biopsy or those that can undergo a longer period between screening approaches.

MS-based approaches to identify serum markers of risk or to facilitate early cancer detection have been studied for some time based on the hypothesis that proteomic panels could identify tumors at an early stage of development. Ovarian cancer has been the subject of several such studies because of diagnosis in late stages, poor patient outcomes, and the absence of a well-established screening method. Two general approaches have been used: identification of distinctive signatures and discovery of discrete markers that might be assembled into panels. Algorithms for ovarian cancer–specific signatures have evolved over time, however, none has been prospectively validated with large numbers of early-stage or preclinical cases. Three serum biomarker panels have been detected from surface-enhanced laser desorption and ionization time of flight (SELDI-TOF) MS peaks representing 14 differentially expressed serum proteins in ovarian cancer, and the combination of four component proteins (transthyretin, β -hemoglobin, apolipoprotein AI, and transferrin) with CA125 has been shown to significantly improve detection of early stage ovarian cancer (54). Once again, these serum biomarker panels require prospective validation.

In the urine, the sensitivity of FISH or of cytokeratin (e.g., keratin 19, 20) detection using immunohistochemistry or reverse transcriptase (RT)–PCR may be higher than that of conventional cytology for bladder and other urothelial cancer screening (50). In one study, a commercial kit (UroVysion) containing hybridization probes for chromosomes 3, 7, 9p21, and 17, was used for FISH analysis of urine. The overall sensitivity and specificity associated with this FISH analysis were 60% and 82.6%, respectively, for detection of bladder cancer. In contrast, the sensitivity and specificity associated with urine cytology were 24.1% and

90.5%, respectively. By T stage, the sensitivity of FISH and cytology, respectively, was 36.1% and 15% in pTa, 65.2% and 25.7% in pT1, and 100% and 66.7% in pT2–3 tumors. By tumor grade (G), similar results were obtained: sensitivity was 37% and 14% in G1, 65.4% and 40% in G2, 91.7% and 50% in G3 disease for FISH and cytology, respectively. This and other studies suggest FISH assay for chromosomes 3, 7, 9, and 17 may have a higher sensitivity than cytology and a similar specificity in the detection of urothelial cancers. Table 25-1 shows the sensitivity, specificity, and positive predictive value of various approaches that have been investigated as potential tools to facilitate bladder cancer screening (55).

Circulating Tumor and Other Cells

Circulating tumor cells (CTCs) have potential utility in cancer screening, target identification for modulation of early carcinogenesis, and in monitoring response to treatment. Indeed, the predictive and prognostic utility of CTCs have already been demonstrated in metastatic breast cancer (56). In these studies, immunomagnetic enrichment (CellSearch, Veridex, LLC) was used to quantify CTCs. Initial studies of the utility of CTCs in cancer screening are now under way. One study assayed a panel of CTC tumor markers in women with suspicious mammograms. The CTCs were captured using magnetic beads coated with a Ber-EP4 monoclonal antibody specific for epithelial cells and this approach was capable of detecting four cells per 10 mL of blood (57). RT-PCR was then used to detect cytokeratin 19, mammaglobin, γ -aminobutyric acid type A receptor (GABA) pi, in addition to two novel genes (B305D-C and B726P). This approach identified 71% of patients subsequently confirmed to have breast cancer. Another early study in ovarian cancer found peripheral blood CTC-specific p53 sequences in some FIGO stage III/IV ovarian cancer patients, suggesting that this approach may be useful as a building block toward early ovarian cancer detection (58).

The use of CTCs to study molecular biomarkers is largely limited to gene expression signatures because of the need for substrate amplification. As most CTCs are undergoing death due to anoikis, robust and stable makers such as DNA copy number or

Table 25-1 Sensitivity, Specificity, and Positive Predictive Value of Urine Cytology, BTA Immunoassay, Detection, ImmunoCyt, and Urine FISH for Early Detection of Bladder Cancer

	Sensitivity (%)	Specificity (%)	PPV (%)
PSA	72	93	25
Urine cytology	48–73	48–100	48–69
BTA	53	77	63
NMP-22	71	66	21
ImmunoCyt	78–81	74–100	26
FISH	69–71	78–95	68

Serum PSA in prostate cancer screening is shown only as a point of reference. ImmunoCyt is approved by the U.S. Food and Drug Administration for the monitoring of recurrent bladder cancer. ImmunoCyt uses a cocktail of three monoclonal antibodies to detect bladder cancer cells in the urine. One antibody is directed against a high-molecular-weight form of glycosylated carcinoembryonic antigen, 19A211. The other two antibodies, LDQ10 and M344, are directed against mucins that are specific for bladder cancer and are labeled with fluorescein. BTA, bladder tumor antigen; FISH, fluorescence in situ hybridization; NMP-22, nuclear matrix protein-22; PPV, positive predictive value; PSA, prostate-specific antigen. Source: Araki M, Nieder AM, Manoharan M, et al. Lack of progress in early diagnosis of bladder cancer. *Urology* 2007;69:270–274.

mutations may be preferable biomarkers. CTCs have the potential to not only facilitate early detection but also can provide access to tumor genome and proteome for novel molecular studies of biomarkers and therapy targets in early stage cancer (59,60). CTCs may ultimately facilitate therapy targeting before definitive treatment, treatment monitoring, and perhaps even allow some patients to avoid surgery. A major challenge is the difficulty in harvesting CTCs and exploring molecular markers in a limited number of cells. Alternative methods of enrichment of CTCs are being explored and include the enhanced density gradient system (61). We are investigating novel methods of DNA/protein extraction to allow detection of mutations and protein expression/activation changes in CTCs, the latter utilizing reverse-phase protein arrays (62–64). Circulating endothelial cells (CECs) and bone-marrow–derived endothelial precursor cells play an important role in tumor neovascularization and growth and, although largely studied thus far for a role in therapy monitoring, also have potential to yield insights into the early origin and pathogenesis of different cancers (65–67).

Novel Molecular Imaging Approaches

Novel molecular imaging approaches such as positron emission tomography (PET) are making feasible the potential incorporation of important and novel cancer molecular markers and signaling pathways into imaging strategies to enhance early detection and treatment monitoring. It is theoretically possible to image any potential cancer-specific marker or target *in vivo* by selecting and labeling an appropriate ligand (e.g., radiolabeled fluoroestradiol [FES]–PET; 68). Novel CT and magnetic resonance imaging (MRI)–based techniques have refined imaging of other cancer-specific processes such as angiogenesis. Such technologies are new, and the most feasible and appropriate approaches to their application to early cancer detection and in the monitoring of treatment responses remain to be defined.

Challenges in Validation of Novel Molecular Screening Biomarkers

A major concern for the application of discovery technologies to molecular cancer screening is the reproducibility of findings, particularly with comprehensive high-throughput approaches to molecular profiling. With the use of novel technologies to profile the genome or proteome for the purpose of defining biomarker panels associated with clinical endpoints such as early tumor detection, the limitations in reproducibility are in large part attributable to the simultaneous assay of many gene or protein markers with a limited number of cancer specimens. The resultant large number of potential biomarker combinations introduces a significant likelihood that uncovered associations are simply the result of chance (multiple-parameter problem). It is therefore critical to adopt a rigorous training, test, and validation approach to screening studies if they are to have a meaningful impact on patient management. Molecular studies using novel high-throughput approaches to analysis of DNA, RNA, and protein biomarkers have advanced

more in the area of treatment response prediction than early cancer detection (69,70). Studies that apply these approaches to molecular cancer screening have much potential but must learn from mistakes that have been made in the evaluation of novel predictive biomarker panels. Most studies that have attempted to define novel predictive biomarker panels have not affected patient management because they did not rigorously attempt to deal with the multiple-parameter problem or did not adopt sufficiently robust statistical approaches to independent validation.

Rigorously validated panels of molecular markers are required before their implementation as tools for early cancer molecular screening. Several examples of preliminary panels exist (71,72), but their applicability is still being validated. A major hindrance to the design of novel molecular studies for screening biomarker discovery and validation is the frequent lack of availability of adequately preserved and appropriately annotated samples in large numbers that can be mated to specific technologies. In particular, novel high-throughput approaches are often limited in their utility to fresh-frozen specimens (paraffin-embedded samples are much more plentiful). Thus, a popular model for biomarker development has been discovery using novel high-throughput profiling technologies (e.g., transcriptional profiling) in frozen tissue, followed by validation using moderate-throughput technologies (e.g., RT-PCR) applied to paraffin-embedded tissue. It is critical to define the specific tissue type in which a molecular marker will be validated for use (73,74). Novel comprehensive approaches to biomarker discovery using proteome—or genome—wide expression profiling have now begun to redefine ways in which tumor banks collect and store tumor samples, with a major emphasis currently placed on snap freezing (75,76).

It is not possible to apply all available investigative technologies to every person, or even every patient at high risk for the development of cancer. Even putting cost issues aside, one challenge is to obtain adequate material from biopsy, cellular, or serum specimens. For microarray (transcriptional or CGH) analysis, several hundred nanograms of genomic DNA or RNA are required. However, with the cellular equivalent of a fine-needle aspirate (FNA), the amount of DNA and RNA is in picograms. In the case of 1 to 5 CTCs in 10 mL of blood, the yield of DNA and RNA is even smaller. Amplification by PCR can increase mRNA yield but PCR-generated mistakes are not uncommon. Further, direct amplification of protein is not possible and, although it is feasible to translate mRNA *in vitro*, it is doubtful that this will significantly enhance protein yield and certainly will not allow assessment of posttranslational protein modifications or activation. Sensitive methods such as MALDI-TOF-MS and post-hybridization signal amplification in, for example, reverse-phase protein lysate arrays do magnify detection limits. However, these major difficulties need to be addressed to facilitate application of novel molecular technologies to cancer screening.

Recommendations

An effective program to discover screening (and other) cancer biomarkers will require a collaborative approach, appropriate human tissue sample sets, an integrated informatics platform,

identification and quantitation of candidate biomarkers in disease tissue, mouse models of disease, standardized reagents for analyzing candidate biomarkers in bodily tissues and fluids and implementation of automation. As revealed by The Human Genome Project, standards, reagents, tools, and automation are absolutely necessary for advancement in biomarker development. Indeed, the Microarray Gene Expression Data (MGED) Society has now established standards for presenting and exchanging gene microarray data (Minimum Information about a Microarray Experiment [MIAME]) and plans to expand these standards to other high-throughput genomic and proteomic technologies in the future. With proteomic biomarkers in particular, technology improvements for better fractionation of the proteome, selection of specific biomarkers from complex mixtures, and multiplexed assay of biomarkers would also greatly enhance progress. A fully integrated proteomic biomarker discovery program will therefore center on core resources for technology development and assessment, tissue sample set handling and storage, reagents and informatics (77). New technologies will be evaluated through pilot projects and “biomarker mines” composed of individual investigators and smaller research teams. Upon standardization by the cores, new proteomic technologies can be tested in the clinical setting by cancer site teams dedicated to biomarker discovery at a particular cancer site (Figure 25-6).

Comprehensive tumor banks will be necessary with appropriately preserved specimen sets (frozen and paraffin-embedded) with matched plasma, serum, and urine that are assembled to allow specific probing with conventional and novel molecular technologies for the purposes of discovering and validating screening biomarkers. Importantly, prospectively collected plasma, serum

and urine sets may be necessary for identification and validation of markers. Indeed, it is likely that useful biomarkers will be discovered from the most valuable and rare prospective sample sets, questioning whether these sets should optimally be available for discovery. As studies characterize tissues using high-throughput genomic, transcriptional, and proteomic approaches for biomarker discovery, it is critical that adequate and centralized computational infrastructure and bioinformatic and biostatistical support be developed to allow storage and utilization/integration of the vast and highly heterogeneous data derived from novel “omics” technologies (78). Such a computational and tissue resource should be made available to all investigators in a manner that is easy to use but also protects confidentiality to avoid duplication of efforts. In addition, these resources should facilitate data mining, retrieval, and automatic analysis with readily available statistical software packages such as R or MATLAB, thus allowing automatic high-throughput data integration across genomic, transcriptional, and proteomic platforms and between data sets, as well as analysis of the association of specific aberrations and changes with clinical endpoints (e.g., early cancer detection). Repeated updating should be facilitated as novel “omics” technologies are continually introduced and upgraded. Access would also allow and encourage novel bioinformatic and biostatistical approaches that further our ability to work with and integrate large amounts of data across multiple platforms so as to advance understanding of the pathogenesis of and ability to detect early cancer.

Screening biomarker selection from data derived using novel high-throughput technologies needs careful consideration. New and increasingly robust approaches to biomarker selection include ‘extreme’ sample training (extreme in terms of the clinical endpoint),

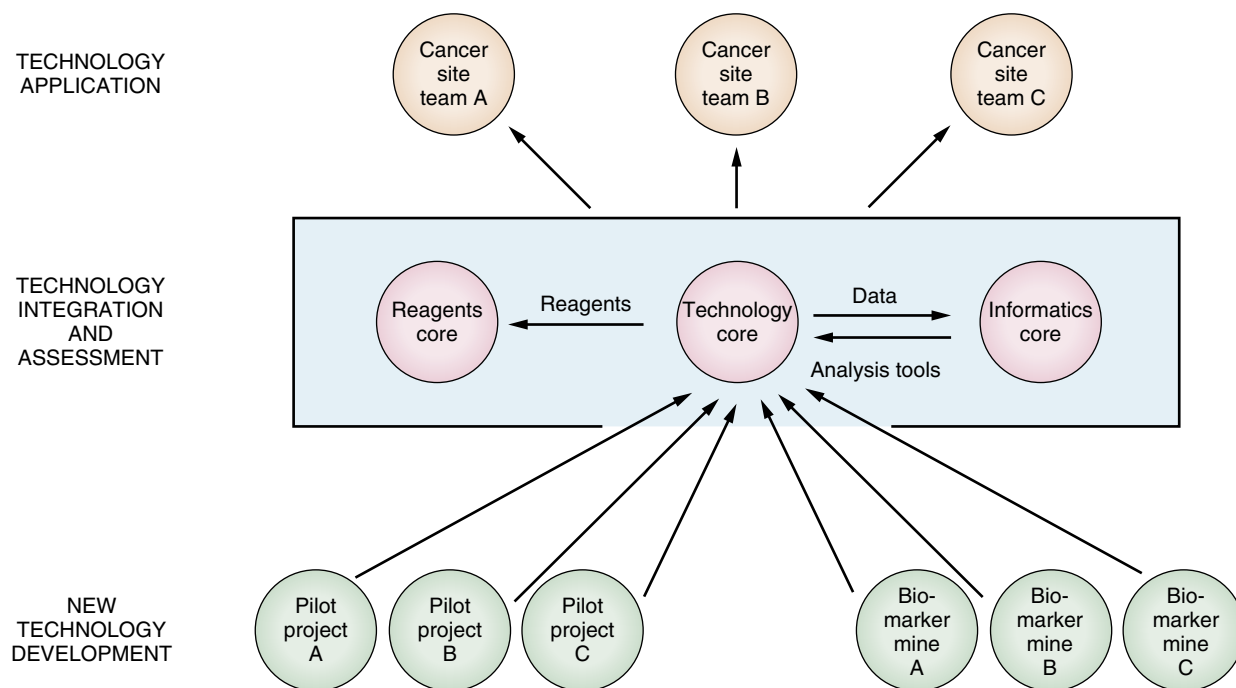


FIGURE 25-6 A fully integrated clinical proteomic biomarker discovery technology program centers on core resources for technology development and assessment, reagents, sample set handling, and storage and informatics. New technologies will be evaluated through pilot projects and “biomarker mines” composed of individual investigators and smaller research teams. Upon standardization by the cores, new proteomic technologies can be tested in the clinical setting by cancer site teams dedicated to biomarker discovery at a particular cancer site. (From Segal E, Friedman N, Kaminski N, Regev A, Koller D. From signatures to models: understanding cancer using microarrays. *Nat Genetics* 2005;37[Suppl]:S38–45, with permission.)

dimension reduction-based penalized logistic regression, cancer outlier profile analysis and multinomial probit regression with Bayesian gene selection (79–82). New biomarkers should ideally be assessed for potential introduction into cancer screening (i.e., for validation) by measuring the impact of the biomarker on survival and costs in a prospective, randomized experiment (83). However, study costs and regulatory and healthcare market constraints often make such clinical trials impractical. Researchers and policymakers are thus increasingly using simulation modeling to predict the effects of new biomarkers on outcomes. The latter approach can optimize sensitivity, specificity, and cost in addition to identifying leverage points where more definitive biomarkers may be needed. Mathematical models can simulate spatial and cellular tumor growth and are often calibrated to observed data from longitudinal population studies of tumor growth and clinical disease. Real or hypothetical screening tests can then be introduced into the model, with sensitivity and specificity calculated on the basis of their ability to detect markers that increase in the blood in proportion to tumor burden, their resolution for detecting tumors of a particular size, or their ability to detect metabolic changes. Based on known or hypothesized distributions representing rates of tumor initiation and growth, the models are then run for populations with and without disease to generate life histories in the presence and absence of testing. The tumor stage at diagnosis as well as disease specific and overall survivals and cost effectiveness can then be compared with and without the screening test. This approach has already been used to assess the cost-effectiveness of flexible sigmoidoscopy for colorectal cancer screening.

Recently, a Committee on Developing Biomarker-based Tools for Cancer Screening, Diagnosis and Treatment of the Institute of Medicine of the National Academies put together a formal set of recommendations for development of biomarker-based tools for cancer and these recommendations are shown in Box 25-3.

Conclusion

In summary, significant progress has already been made in the prevention and early detection of certain forms of cancer, including breast and colorectal cancers. Such progress has largely been based on increasing population awareness of the importance of screening and advances in radiologic imaging in addition to a limited role for molecular markers. However, molecular studies have revealed the biologic heterogeneity and complexity of cancer that necessitates the application of more global studies of the cancer genome and proteome to facilitate further advances in understanding, treatment and early detection. Indeed, the application of high-throughput technologies such as transcriptional profiling is already significantly advancing the field of cancer therapy prediction. Improving understanding of the molecular heterogeneity of cancer along with rapid improvements in molecular technologies that can profile this heterogeneity has also unearthed increasing possibilities for the early detection and prevention of cancer. It is expected that these approaches will eventually not only advance our ability to diagnose cancer at an early stage but will also allow simultaneous profiling of molecular targets that will facilitate individualization of patient care to a point where current standard therapies in many cases may become obsolete. However, the achievement of these goals necessitates overcoming many challenges that lie in the way of the successful implementation of both traditional and novel molecular technologies to cancer screening. Further, as high-throughput technologies acquire an increasing foothold in early detection of cancer and in cancer treatment prediction, the development of robust collaborations and bioinformatics approaches to high-throughput data storage, integration, and analysis will become increasingly necessary. Ultimately, if screening is to be able to detect abnormalities that exist when only a few cells are affected, a practical and cost-effective integrated approach that is capable of comprehensively examining

Box 25-3

Summary of Recommendations to Develop Biomarker-Based Tools for Cancer^a

Methods, Tools, and Resources Needed to Discover and Develop Biomarkers

1. Federal agencies should develop an organized, comprehensive approach to biomarker discovery and foster development of novel technologies.
2. Industry and other funders should establish international consortia to generate and share precompetitive biomarker data.
3. Funders should place a major emphasis on developing pathway biomarkers to broaden applicability.
4. Funders should sponsor demonstration projects to develop biomarkers that can predict efficacy and safety in patients for drugs already on the market.
5. Government agencies and other funders should sustain support for high-quality biorepositories of prospectively collected samples.

Guidelines, Standards, Oversight, and Incentives Needed for Biomarker Development

6. Government agencies and other stakeholders should develop a transparent process to create well-defined consensus standards and guidelines for biomarker development, qualification, validation, and use.
7. The U.S. Food and Drug Administration (FDA) and industry should work together to facilitate the codevelopment and approval of diagnostic–therapeutic combinations.
8. The FDA should clearly delineate and standardize its oversight of biomarker tests used in clinical decision making.
9. The Centers for Medicare and Medicaid Services should develop a specialty area for molecular diagnostics under the Clinical Laboratory Improvement Amendments (CLIA).

Methods and Processes Needed for Clinical Evaluation and Adoption

10. The Centers for Medicare and Medicaid Services should revise and modernize its coding and pricing system for diagnostic tests.
11. The Centers for Medicare and Medicaid Services, as well as other payors, should develop criteria for conditional coverage of new biomarker tests, and as a component of conditional coverage, establish procedures for high-quality population-based assessments of efficacy and cost-effectiveness of biomarker tests.

^aFrom Cancer Biomarkers: The promises and challenges of improving detection and treatment. Committee on Developing Biomarker-based tools for cancer screening, diagnosis, and treatment. SJNASS and HL Moses.

the transcriptome, genome and proteome may become necessary. Techniques already exist that can extract genetic material from single cells and proteomic techniques are able to detect protein at the femto- and attomole levels. The challenge is to identify the individual or panels of biomarkers that are easiest to detect while at the same time being informative and cost-effective for everyday screening. Although laboratory techniques can now analyze biomolecules from almost single cells, they are far too labor-intensive and time-consuming for application to routine clinical practice.

High-throughput technologies must be developed or refined so as to become practical enough for clinical use. Retrospective evaluation of biomarkers can be facilitated by the support and implementation of biorepositories with serum, plasma, and urine specimens from screening trials. Once the most sensitive biomarker panels have been defined, rigorous prospective evaluation will be required. Thus, development of effective strategies for early detection will require well-integrated efforts in the laboratory and the development of relevant and efficient clinical trials.

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Lymphoma/Myeloma

Lymphoid tumors include most (85%) hematopoietic neoplasms (1). In 2006 more than 90,000 new cases of lymphoid malignancies were anticipated in the United States representing the fourth most common cancer in either sex (1), with more than 35,000 patients expected to die of these diseases. Since the 1950s, the incidence of lymphoid malignancies has increased by more than 50% in the United States, with potentially a slight reduction on the late 1990s. This dramatic increase could be partly, but not completely, explained by the refinement of diagnostic tests over time, as well as the emergence of the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) pandemic in the early 1980s.

Lymphoid malignancies include Hodgkin lymphomas (HLs) and non-Hodgkin lymphomas (NHLs), plasma cell disorders, mostly represented by multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia (ALL; Figure 26-1). To overcome the limitations of old classification systems based on morphology, the World Health Organization (WHO) has introduced a classification scheme of lymphoid malignancies that integrates several parameters, including lineage of origin (B or T cells) and the degree of differentiation exhibited by the tumor cells, based on the assumption that the tumor cells of most lymphoid tumors correspond to specific stages of normal lymphoid development (2–4; Tables 26-1 and 26-2). More than 90% of lymphoid tumors are derived from B lymphocytes (Table 26-2). Moreover, mature neoplasms account for more than 90% of all lymphoid neoplasms and comprise lymphomas, plasma cell neoplasms, CLL, and various other known or unknown types (Figures 26-1, 26-2, and 26-3).

Lymphocyte B- and T-Cell Development

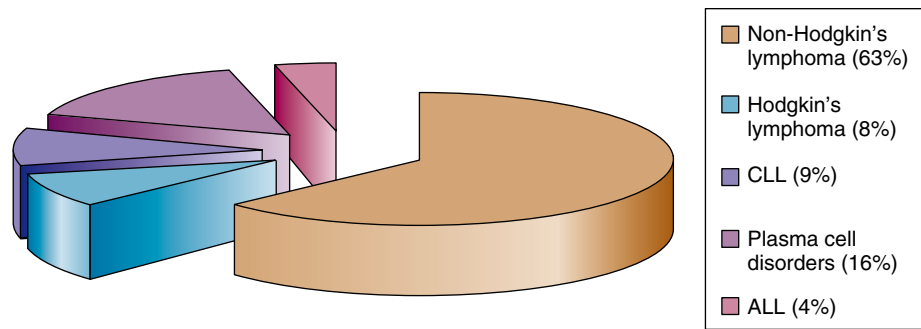
B and T lymphocytes originate from pluripotent hematopoietic stem cells located in the hemopoietic tissues (liver and then bone marrow in the fetus and bone marrow in the adult). T cells develop in the thymus from precursor cells that migrate from the hemopoietic tissues via the blood. Thymus and hemopoietic tissues are referred to as primary lymphoid organs, from which mature B and T cells migrate via the blood to the secondary lymphoid organs (lymph nodes, spleen, and epithelium-associated lymphoid tissues) to eventually interact with foreign antigens.

B lymphocytes undergo a multiple-step evolution during development, accompanied by changes on the expression of cell-surface proteins, differences in functional activity, and rearrangements of the immunoglobulin genes (5,6; Figure 26-3). These stages occur before and after birth in the bone marrow and are completed in the peripheral lymphoid organs. The first identifiable B-cell precursors, based on the presence of the B-cell-specific marker B220, are the pre-pro-B cells, which do not have any rearrangement on the immunoglobulin gene. In the pro-B cells, the V(D)J recombination process, mediated by RAG1 and RAG2, next induces DNA double-strand breaks and assembles all the different components of the final B-cell receptor (BCR), the immunoglobulin that is expressed on the surface. When B cells reach this stage of immature naive B cells, they migrate to the peripheral organs, where they undergo additional genetic and phenotypic modifications. Specifically, they undergo somatic hypermutation to increase their affinity for the antigen and finally switch recombination, from IgM they become IgG and less frequently IgA. Once they undergo this process, they exit and become plasma cells or memory B cells. It is widely believed that the presence of these three physiologic mechanisms of DNA remodeling are linked to the occurrence of chromosomal translocations and somatic mutation in B-cell lymphoid tumors and are associated with the high frequency of tumors seen in this group when compared with T-cell lymphoid malignancies (5,6). Indeed, T lymphocytes undergo similar processes affecting the T-cell receptor, but fewer genetic rearrangements are ongoing in T cells.

General Concepts about the Pathogenesis of Lymphoid Malignancies

The presence of hallmark chromosomal translocations in hematologic malignancies in general, and specifically in lymphoid cancers, has been recognized for more than 40 years. It is important to note, however, that the pathogenetic role for most of these genetic lesions remains for the most part speculative. In general, these chromosomal translocations are not sufficient by themselves to induce cancer, and other poorly defined genetic or epigenetic lesions are required for transformation. A case in point is represented by the chromosomal translocation t(14;18) in follicular lymphoma

FIGURE 26-1 Distribution of lymphoid malignancies. ALL: acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia. (From Harris NL, Jaffe ES, Diebold J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol* 1999;17:3835–3849, with permission.)



that dysregulates the anti-apoptotic gene *BCL2*. Overexpression of *BCL2* is not sufficient to confer tumorigenicity to human B-lymphoblastoid cell lines, but the cooperation of *BCL2* with another oncogene (e.g., *MYC*) is able to induce transformation (7). Moreover, patients with t(14;18) do not always have *BCL2* overexpression and long-lived clones harboring the oncogenic t(14;18) chromosomal translocation are commonly present in normal humans, rise in frequency with age, and have no clinical sequelae.

The search for other genetic lesions, other than chromosomal translocations, as for example point mutations and epigenetic modifications (see subsequent sections), has been successful in

several cases. Indeed, the genome of a tumor cell, either of hematologic, epithelial or mesenchymal origin, appears so deeply rearranged that the genetic events responsible for initiation and for the molecular evolution of lymphoid malignancies are for the most part unknown.

Expression profiling has yielded interesting insights into molecular pathways that could be important in the pathogenesis of these diseases. Based on these findings, research has identified pathways that are essential in the pathogenesis of these diseases, prominent among them the NF- κ B pathway. In most cases, the genetic lesions determining the aberrant expression of some or all

Table 26-I World Health Organization Classification of Lymphoid Tumors

B-cell neoplasms	Mature (peripheral) T-cell neoplasms
Precursor B-cell neoplasm	T-cell prolymphocytic leukemia
Precursor B-lymphoblastic leukemia/lymphoma (precursor B-cell acute lymphoblastic leukemia)	T-cell granular lymphocytic leukemia
Mature (peripheral) B-cell neoplasms	Aggressive NK-cell leukemia
B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma	Adult T-cell lymphoma/leukemia (HTLV11)
B-cell prolymphocytic leukemia	Extranodal NK/T-cell lymphoma, nasal type
Lymphoplasmacytic lymphoma	Enteropathy-type T-cell lymphoma
Splenic marginal zone B-cell lymphoma (½ villous lymphocytes)	Hepatosplenic gamma-delta T-cell lymphoma
Hairy cell leukemia	Subcutaneous panniculitis-like T-cell lymphoma
Plasma cell myeloma/plasmacytoma	Mycosis fungoides/Sezary syndrome
Extranodal marginal zone B-cell lymphoma of MALT type	Anaplastic large-cell lymphoma, T/null cell, primary cutaneous type
Nodal marginal zone B-cell lymphoma (½ monocytoid B cells)	Peripheral T-cell lymphoma, not otherwise characterized
Follicular lymphoma	Angioimmunoblastic T-cell lymphoma
Mantle-cell lymphoma	Anaplastic large-cell lymphoma, T/null cell, primary systemic type
Diffuse large B-cell lymphoma	Hodgkin'S lymphoma (Hodgkins disease)
Mediastinal large B-cell lymphoma	Nodular lymphocyte-predominant Hodgkin's lymphoma
Primary effusion lymphoma	Classic Hodgkin's lymphoma
Burkitt lymphoma/Burkitt cell leukemia	Nodular sclerosis Hodgkin's lymphoma (grades 1 and 2)
T-cell and NK-cell neoplasms	Lymphocyte-rich classic Hodgkin's lymphoma
Precursor T-cell neoplasm	Mixed cellularity Hodgkin's lymphoma
Precursor T-lymphoblastic lymphoma/leukemia (precursor T-cell acute lymphoblastic leukemia)	Lymphocyte depletion Hodgkin's lymphoma

Only major disease categories are listed; subtypes and variants are discussed in detail in Jaffe ES, Harris NL, Stein H, Vardiman JW (eds.). *World Health Organization Classification of Tumors: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon, France: IARC Press, 2001.
HTLV11, human T-cell leukemia virus; MALT, mucosa associated lymphoid tissue; NK, natural killer.
Source: Harris NL, Jaffe ES, Diebold J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting. Airlie House, Virginia, November 1997. *J Clin Oncol* 1999;17:3835–3849, with permission.

Table 26-2 Lymphoma subtype derivations

Lymphoma Subtypes	Immunophenotype	Frequent Translocations	Genes Involved	SHM	Ongoing SHM	Putative Cell of Origin
GC B-cell-like DLBCL Activated B-cell-like DLBCL	CD20, CD22, CD79a, BCL-2	t(3;*) (q27;*) → t(14;18) → t(8;14) →	BCL6 (35%) BCL2 (15%–30%) MYC (15%)	✓ ✓	✓ —	GC B cell GC B-cell subset or extra-GC mutated B cell
Follicular lymphoma (FL)	CD20, CD22, CD79a, CD10, BCL-2, BCL-6	t(14;18) →	BCL2 (90%)	—	—	GC B cell
MALT lymphoma	CD20, CD79a; Negative for CD5, CD10, CD23	t(11;18) → t(1;14) →	API2-MALT1 (30%) BCL10 (5%)	✓	✓	GC B cell or post-GC B cell
Mantle-cell lymphoma	CD20, CD79a, CD5; Negative for CD10, CD23	t(11;14) →	Cyclin D1 (95%)	—	—	Pre-GC B cell
Burkitt lymphoma	CD20, CD10, BCL-6	t(8;14) →	MYC (100%)	✓	✓	Pre-GC B cell
Multiple myeloma	CD38, CD138	t(11;14) → t(4;14) → t(14;16) → t(6;14) → t(14;20) →	Cyclin D1 (15%–20%) MMSET-FGFR3 (12%) MAF (5%–10%) Cyclin D3 (5%) MAFB (5%)	—	—	Post-GC B cell
Hodgkin's lymphoma (classic type)	CD30, CD15 (CD79a)	—	—	✓	—	GC or post-GC B cell
Hodgkin's lymphoma (nodular lymphocyte predominant type)	CD20, CD79a, BCL-6, CD45, CD75.	—	—	✓	✓	GC B cell

GC, germinal center; DLBCL, diffuse large cell lymphoma; MALT, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissues; SHM, somatic hypermutation.

the genes in any specific pathway have not yet been identified. For example the NF- κ B pathway is often dysregulated in cancer but only a handful of underlying genetic lesions have been identified.

In addition to genetic or epigenetic lesions driving tumor proliferation and progression, two other components have emerged as essential in lymphoid tumorigenesis in recent years: the presence and activation of the B receptor on the surface of B-cell lymphoma cells, and the interactions of the cancer cells with their surrounding microenvironment.

The presence of an active BCR on the surface of developing and mature B cells is essential for their survival. Indeed, most B cells undergo apoptosis during development in the bone marrow and during their final maturation in the peripheral lymphoid organs due to expression of a BCR that is directed against self or possesses a low affinity toward antigen. The process of somatic hypermutation, which increases the affinity of the BCR for its antigen, allows for the subset of B cells having a BCR with high affinity for antigen to survive. This dependency on BCR is present in mature resting B cells as well: Ablation of the BCR in mice led to rapid death of mature B lymphocytes, delayed by constitutive bcl-2 expression (8). Circumstantial evidence suggests the relevance of the BCR receptor and its activation in at least a subset of lymphomas. Despite the frequent occurrence of oncogenic translocations involving the IgH locus, most B-cell malignancies

express surface immunoglobulin. It has been shown that most IgH translocations involve nonproductively rearranged alleles (9), which suggests that the transformed B cell relied on expression of a functional BCR for survival, at least early in the history of the malignant clone. In hepatitis C virus (HCV)–associated lymphomas, HCV-specificity of BCR has been reported in some cases, and disease regression occurs after antiviral therapy (for a more complete discussion on this topic, see [5]). Gastric mucosa-associated lymphoid tissue (MALT) lymphomas are reactive to *Helicobacter pylori* (*H. pylori*), and antibiotic treatment of the infection often induces a reduction on the disease (see following sections). In this case, the effect seems to be mediated by T-helper cells that recognize the bacteria and in turn activate proliferation in a B-cell–specific population.

The second component that has an important role in tumor cell survival and proliferation is represented by the microenvironment, particularly in mature lymphomatoid tumors, as for example follicular lymphoma where tumor cells can proliferate in vitro only if co-cultured with CD4⁺ T cells or with stromal cells and an antibody against the CD40 receptor (10). Moreover, the length of survival among patients with follicular lymphoma correlated with the molecular features of nonmalignant immune cells present in the tumor at diagnosis (11). A similar pattern of growth depending on CD40 and stromal cells has been suggested for B-CLL, and

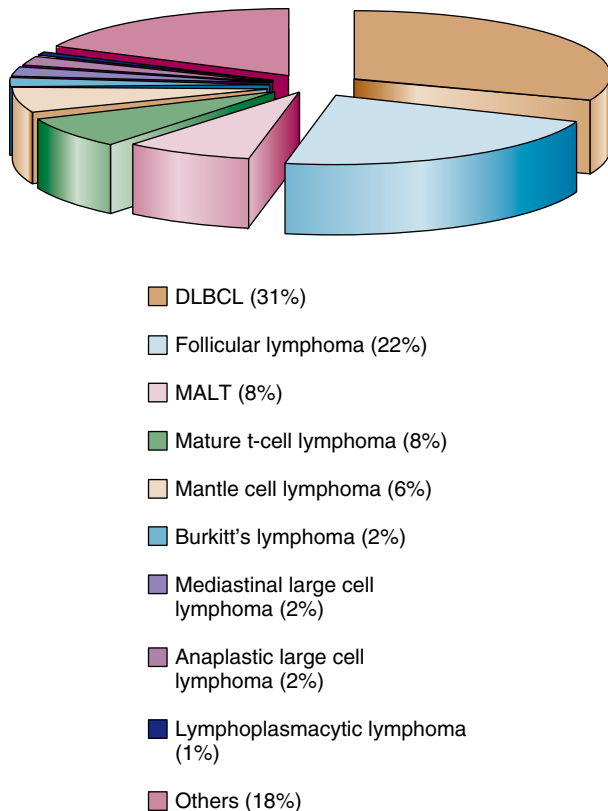


FIGURE 26-2 Distribution of non-Hodgkin's lymphomas. DLBCL, diffuse large cell lymphoma; MALT, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissues. (From A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. Blood 1997;89:3909–3918, with permission.)

MM malignant plasma cells survive only within the context of a net of cytokines and adhesion molecules present in the bone marrow microenvironment (see following sections).

Molecular Mechanisms of Lymphoid Malignancies

Several genetic and epigenetic mechanisms have been implicated in the activation of oncogenes or the inactivation of tumor suppressor genes in lymphoid malignancies. Among the mechanisms that have been more extensively studied, chromosomal translocations occupy a prominent role. The translocations occurring in mature B-cell malignancies are usually represented by reciprocal translocations that are recurrent within a specific disease (typically, mature T-cell malignancies do not present hallmark chromosomal translocations). In contrast with translocations that are present in the immature form of lymphoid malignancies, they tend to juxtapose oncogenes to regulatory sequences derived from other genes in partner chromosomes and are not the result of fusion between two different genes with the formation of chimeric genes. These translocations therefore result in dysregulation in the expression of the oncogene driven by a different promoter. In some cases, the target gene is expressed by the normal cellular counterpart from which the tumor derives, but after the translocation the level of

expression becomes much higher. In other cases, the oncogene is not expressed in the normal cells from which the tumor derives, and therefore it is the inappropriate expression of the gene that causes or promotes the tumor. The chromosomal translocations in mature lymphoid malignancies are likely the aberrant outcome of genomic rearrangements that occur otherwise physiologically during the development of normal lymphoid tissues, in particular on the B-cell compartment, as mentioned previously.

Other mechanisms determining the activation of oncogenes include amplifications (e.g., the *REL* gene) and point mutations that alter the coding sequence (as in the case of *MYC* or *BCL2*) or the regulatory regions (as in the case of *MYC* or *BCL6*). Interestingly, mutations of the different forms of *RAS* are not a frequent event in lymphoid malignancies, with the exception of multiple myeloma; in contrast, this gene family is commonly mutated in epithelial carcinomas. Biallelic inactivation of tumor suppressor genes has been reported for lymphomas, usually with concomitant hemizygous deletion of one copy associated with mutation in the remaining copy of the gene. Among the tumor suppressor genes inactivated in lymphoid malignancies are *TP53*, *CDKN2A* (p16), *PTEN*, *BAX*, *CDKN2C*, and *ATM* (see following sections).

Two herpes viruses have demonstrated a role in lymphomagenesis. The Epstein Barr virus is present in nearly all endemic Burkitt lymphoma, many post-transplant and primary effusion lymphomas, and about 40% of cases of classical HL. The mechanism by which EBV drives lymphomagenesis is mediated by EBV-encoded proteins that activate key signaling pathways in B cells: Latent membrane protein-1 (LMP1) mimics an active CD40 receptor; LMP2A activates the BCR pathway; and EBV nuclear antigen 2 (EBNA2) signals in a manner similar to the Notch pathway (12).

The second herpes virus that could induce lymphomas is human herpes virus-8, implicated in the pathogenesis of primary effusion lymphomas. The oncogenic mechanism partly relies on the ability of the virus to activate the transcription factor NF- κ B.

Finally, human T-lymphotropic virus-1 is associated with peripheral T-cell lymphoma. Cells infected with this virus express a protein called TAX that is considered the major cause of the tumor, albeit no clear mechanism has yet been identified.

Molecular Pathogenesis of Non-Hodgkin's Lymphomas

Detailed information on the immunophenotype, most recurrent chromosomal translocations and genetic lesions, and presence of somatic hypermutations and putative cells of origin for the most frequent lymphoid malignancies are reported on Table 26-2. More than 85% of NHL derives from B cells, specifically from germinal center (GC)-like or post-GC-like cells (13).

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma represents the most frequent type of NHL. Many genetic lesions are characteristic of this tumor through different mechanisms: chromosomal translocations (14) affecting

ACUTE LYMPHOBLASTIC LEUKEMIAS

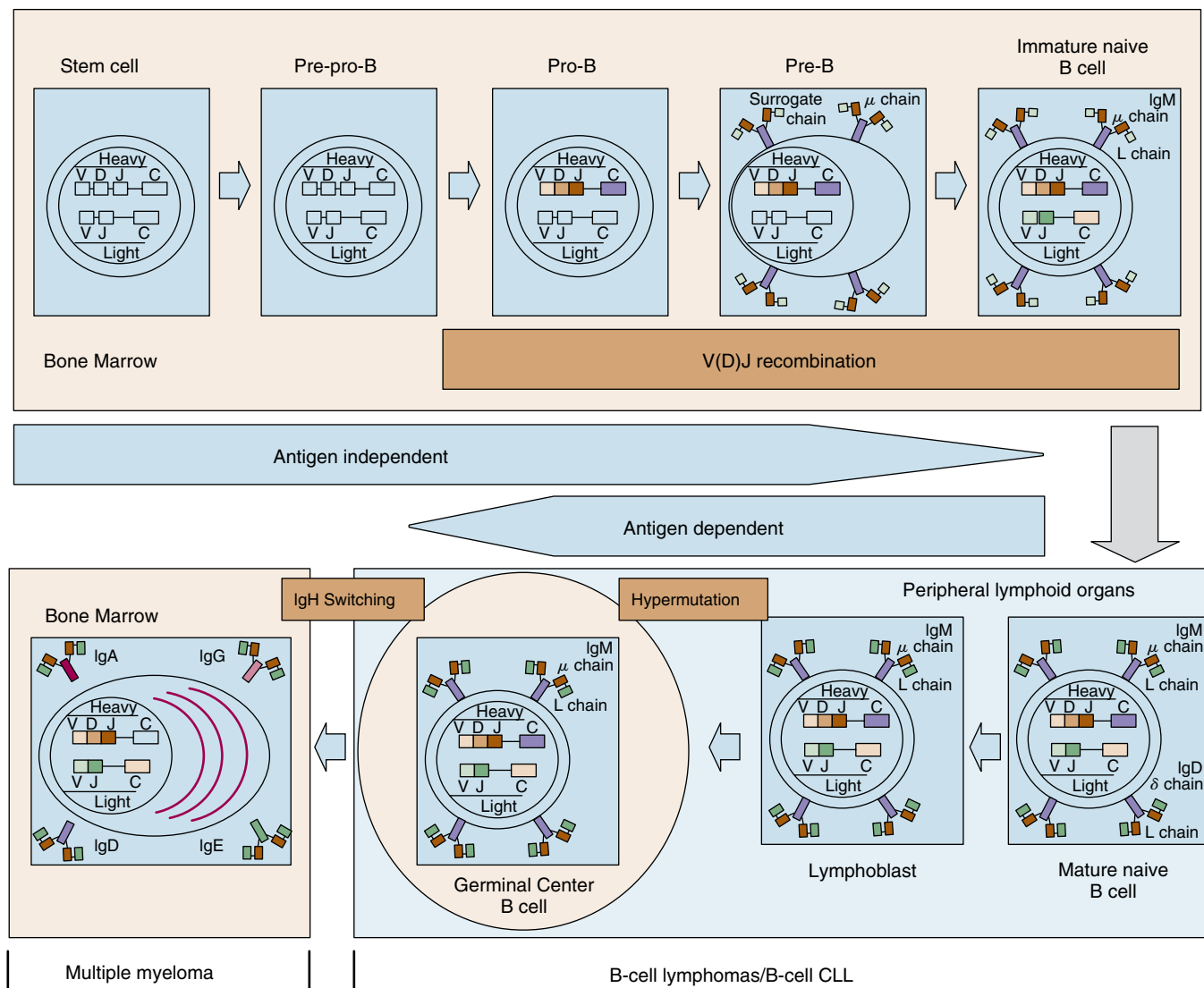


FIGURE 26-3 STAGES OF B-CELL DEVELOPMENT AND CORRELATION WITH DIFFERENT LYMPHOID DISEASES. The top panel shows the different stages of early development of B cells within the context of the bone marrow, before the interaction of the B cell with the antigens. Cancers arising in these cells belong to the group of acute leukemias. After the precursor, naive B cells leave the bone marrow and move to the peripheral lymphoid organs and undergo the final stages of maturation and encounter antigens. Cancers arising in these cells belong to the group of lymphomas. After antigen stimulation, mature B cells evolve into plasma cells that leave the peripheral lymphoid organs and home back to the bone marrow. Myeloma represents a cancer arising in plasma cells (see text for additional information).

BCL6 (30%–40% of cases), *BCL2* ($t(14;18)$; 17%–20%), *MYC* ($t(8;14)$; 6%); genomic amplifications involving *BCL2*, *REL*, and *MYC*; and somatic hypermutation (*BCL6*, *PIM1*, *MYC*, *PAX5*, *RHOH*). The tumor suppressor gene *TP53* is mutated in approximately 20% of cases.

Recent expression (15) and genetic analysis have highlighted how this tumor encompasses at least two different subtypes: the GC B-cell-like DLBCL arising from the GC; and the activated B-cell-like DLBCL arising from a GC B-cell subset or extra-GC mutated B cells. Based on the mutation status of the variable region of the Ig gene, the GC B-cell-like group shows ongoing somatic hypermutation (different mutations are present in different clones of the tumor) whereas all the tumor cells in the second group share

the same mutation. Interestingly, a significantly different outcome between the two groups has been shown, with the GC-like group showing a more favorable prognosis versus the post-GC DLBCL (65% vs. 35% survival at 5 years, respectively). This distinction underscores differences in the presence and incidence of genetic lesions and carcinogenic pathways between the two groups, suggesting at least a partially distinct molecular pathogenesis. For example, *BCL2* translocations and amplification of *REL* are uniquely present in the GC group, whereas *NF- κ B* activation is a hallmark of activated B-cell-like DLBCL.

BCL6 plays a prominent role in DLBCL. In 30% to 40% of the cases, reciprocal chromosomal translocations involving *BCL6* at 3q27 substitute *BCL6* promoter with an heterologous promoter,

most frequently on chromosome bands 14q32, 2p12, and 22q11 corresponding to the promoters of the Ig genes; however, more than 20 alternate loci on other chromosomes have been described. Other lymphomas do not have translocations affecting this gene, with the exception of few cases of follicular lymphomas. *BCL6* gene expression is tightly regulated during B-cell differentiation, being restricted to B cells in the GC. In contrast, the heterologous promoters exhibit a broader spectrum of activity in B-cell ontogenetic stages, thus preventing *BCL6* down-regulation in post-GC cells. An additional mechanism for dysregulating the expression of *BCL6* is through somatic hypermutation of the promoter region, which induces point mutations and microdeletions or amplifications in the 5' region of the gene, in close proximity to the promoter region within the major cluster of chromosomal breaks at 3q27. These lesions occur in approximately 70% of DLBCL, 50% of follicular lymphomas, and 40% of Burkitt lymphomas. Although the functional relevance of these mutations is not completely understood, the cumulative frequency of mutations and rearrangements in DLBCL approaches 100%, suggesting a major role for the dysregulation of this gene in the pathogenesis of DLBCL. *BCL6* is a transcriptional repressor that belongs to the family of the DNA-binding proteins containing a zinc finger domain. Another domain, POZ, is involved in protein–protein interactions. As for the oncogenic mechanism responsible for the disease, *BCL6* exerts an essential role in inducing the formation of GC and in repressing the terminal differentiation of B cells through the inhibition of Blimp1. Another function likely important in tumorigenesis is the increased proliferation of B cells induced by *BCL6*, likely through repression of *CDKN1B* (p27Kip1). Finally, *BCL6* prevents apoptosis after DNA damage, through the down-regulation of *TP53*. Mutations of *TP53* occur in approximately 20% of DLBCL and occur usually in tumors that do not present *BCL6* translocations, supporting the notion that in the presence of *BCL6* overexpression p53 is already down-regulated. It should be emphasized, however, that *BCL6* dysregulation alone is not sufficient to induce tumorigenesis, and other oncogenic lesions are necessary (16).

Other less aggressive lymphomas can evolve into DLBCL, as for example most cases of follicular lymphomas in their later stages (often after acquiring mutation in *TP53*), CLL (in 5% of the cases), and MALT. Indeed, when DLBCL presents with a translocation typical of other NHL, as for example the 20% of DLBCL presenting with t(14;18), it is often an indication of its derivation from other, less aggressive, lymphomas.

Follicular Lymphoma

Virtually all cases of follicular lymphoma (FL) show cytogenetic abnormalities, most prominently t(14;18) involving *BCL2*, which is present in approximately 90% of the cases (17). *BCL2* is an anti-apoptotic protein, and additional genetic lesions are necessary to induce a full-blown lymphoma (see preceding sections). *BCL2* transgenic mice develop polyclonal hyperplasia of long-lived resting B cells, and monoclonal B-cell lymphoma emerges only after a long latency period in the form of aggressive disease characterized

by the acquisition of secondary genomic alterations. Indeed, most human FLs demonstrate at least one additional karyotypic abnormality, on average six, with particularly frequent losses of –1p, –6q, and –17p and +7, +12q, +18q/dup der(18)t(14;18). The *BCL2* gene is translocated to the immunoglobulin heavy-chain gene locus, placing the intact *BCL2* under the influence of the immunoglobulin gene enhancer. *BCL2* is therefore overexpressed and prevents apoptosis, with the consequence of inappropriately rescuing long-lived B cells that would have been otherwise destined to apoptosis in the absence of antigenic selection.

In 10% of the cases of FL no t(14;18) is detectable, but it is not known which lesion could replace t(14;18) in these lesions. *BCL6* is rearranged in ≈15% of the cases and mutated in its regulatory region in up to 40% of the patients, but it is unclear if this lesion could represent an alternative survival signal. It is also not clear which other lesions could be present in this disease and/or represent progression events. Mutations in *TP53* and mutations, deletions, or methylation of *CDKN2A* (p16) have been reported in a subset of patients. The relevance of the microenvironment in the development of FL seems to be particularly significant, especially the role of T cells and dendritic cells (see preceding sections). Intriguingly, more than 90% of FL have areas histologically indistinguishable from DLBCL.

Extranodal Marginal Zone B-Cell Lymphoma of Mucosa-Associated Lymphoid Tissues

MALT originates in lymphoid tissues disseminated in the stomach (70% of cases), bronchial epithelium (14%), ocular adnexa (12%), thyroid (4%), and intestine (1%; 18). Autoimmune or infectious diseases represent a major determinant for this disease, at least in the early stages, as for example the infection by *H. pylori* in the stomach in more than 90% of cases of gastric MALT. The working model to explain the pathogenesis of this disease suggests that *H. pylori* infection, by inducing the accumulation of MALT, is a necessary precursor to the development of gastric MALT lymphoma. The chronic, active inflammation induced by *H. pylori* might cause reactive oxygen species (ROS)–induced DNA damage, leading to various genetic abnormalities and the subsequent emergence of a neoplastic B-cell clone. In later, more aggressive stages, MALT acquire specific chromosomal translocations and mutations that confer a more aggressive behavior to the tumor. Indeed, gastric MALTs could be fully eradicated with antibiotic treatment in approximately half of the cases (usually the cases without genetic lesions), whereas the other half are more advanced cases, with genetic lesions, and do not respond to the therapy. A common feature among the genetic lesions described in MALT is the activation of the NF-κB pathway. For example, in 20% to 25% of MALT cases, t(11;18) has been reported, which results in the fusion between the genes *API2* (member of the inhibitor of apoptosis family IAP that exerts anti-apoptotic effects) and *MLT*, which induces NF-κB activation. Interestingly,

when present, this translocation is the only chromosomal aberration, whereas in t(11;18)-negative cases, multiple chromosomal rearrangements are present. This translocation is associated with adverse clinical features, including dissemination to lymph nodes and distal sites, or unresponsiveness to *H. pylori* treatment. Another reported translocation is t(1;14), which is present in 5% of MALT cases; associated with trisomies of chromosomes 3, 12, and 18; and linked to poor prognosis. It affects patients who do not respond to antibiotic therapy. This translocation juxtaposes the regulatory region of the Ig-heavy-chain gene enhancer with the gene *BCL10*, which links BCR activation with the NF- κ B pathway, and therefore induces proliferation. In a minority of patients, a t(14;18) affecting MALT1 induces its overexpression. This translocation seems to predominate among nongastrointestinal MALT lymphomas.

Mantle Cell Lymphoma

MCL is the most aggressive among the lymphoid malignancies, with a median survival of 4 to 5 years (19). Two main cytologic variants of MCL have been described, namely a classical subtype with small to medium neoplastic cells and a blastoid subtype, associated with a more aggressive clinical behavior and characterized by larger, sometimes more pleomorphic or lymphoblast-like tumor cells.

The hallmark genetic lesion in MCL is the translocation t(11;14), which juxtaposes the IgH enhancer element in front of the intact cyclin D1 gene, thereby driving its overexpression in 95% of the cases. As for other lymphomas, however, additional genetic lesions must occur to induce the tumor. Among these, deletion of the *INK4A* locus, including the two tumor suppressor genes *p16* and *ARF* is present in 20% of the cases. *p16* inhibits CDK4 and CDK6, which cooperate with cyclin D1 in promoting G₁/S transition by phosphorylating and inhibiting RB1. Given the ubiquitous presence of t(11;14) dysregulating cyclin D1 expression in MCL, the concomitant deletion of *p16* is unexpected, since it belongs to the same pathway. However, the relevance of *p16* deletion is suggested by cases with wild-type *p16* where other genes acting in the same pathway, including the oncogenes CDK4 and *BM11*, were amplified and overexpressed; therefore, it is unclear why both cyclin D1 and *p16* are altered in the same tumors.

MCL tumors present a high level of chromosomal rearrangements, suggesting impairment of DNA damage and mitosis checkpoint defects. Indeed, the ataxia telangiectasia mutated (*ATM*) gene presents mutations in 40% to 75% cases of MCL, mainly affecting the PI3K (PI3K, phosphatidylinositol-3-kinase) domain or leading to truncations of the *ATM* protein. The association of such mutations with a higher number of chromosomal rearrangements than in wild-type *ATM* suggests a prominent role of nonfunctional *ATM* in driving genomic instability in MCL. Another gene in this pathway, *CHK2*, has been reported as mutated or down-regulated in a small subset of patients with MCL. Finally, *TP53* is mutated in approximately 30% of the blastoid MCL and is associated with a more aggressive disease.

Burkitt Lymphoma

There are three variants of BL: the endemic form occurs in Africa, is caused by infection with the Epstein Barr virus (EBV), and affects children between 4 and 7 years of age; the sporadic form occurs throughout the world in children and young adults; and finally the immunodeficiency-associated form is related to HIV infection. All three forms are linked to genetic rearrangements affecting the oncogene *MYC*: in 80% of the cases, t(8;14) juxtaposes the enhancer of the IgH gene with *MYC* and in the remaining 20%, *MYC* is under the control of the enhancers of the λ or κ light-chain loci as a result of the translocations t(2;8) and t(8;22), respectively. The multifaceted mechanism by which *MYC* induces lymphoma is not completely understood, but its role as a potent oncogene is firmly established. *MYC* induces telomerase expression, promotes growth and differentiation, blocks apoptosis, induces cell cycle progression, and increases cellular metabolism (for a review on *MYC* and its role in BL, see [20]). Additional genetic events contribute to the progression of the disease, both within the *MYC* sequence (as for example additional mutations that increase the proteasome resistance of *MYC*), or affecting other genes, including mutations in *TP53*, methylation of death-associated protein kinase (DAP-kinase), and down-regulation of *INK4A* (p16) or *INK4B* (p15) via gene hypermethylation. The role of EBV in disease pathogenesis has not been elucidated.

Hodgkin's Lymphoma

The most recent classification of lymphomas (3,4) subdivides HL into two groups: the nodular lymphocyte predominant HL (nLPHL; 5% of the cases); and the classical Hodgkin lymphoma (cHL), which includes the remaining 95% of cases. Within cHL, the disease is further subdivided into four groups: nodular sclerosis, mixed cellularity, lymphocyte depleted, and lymphocyte-rich (the first two encompassing more than 90% of the cases; 21). A hallmark of HL that has hampered the identification of the tumor cell of origin and prevented comprehensive analysis on the genetics of this disease is the very abundant infiltration of the tumor by surrounding normal polyclonal reactive cells, including lymphocytes, macrophages, eosinophils, plasma cells, stromal cells, and fibroblasts; tumor cells represent only 1% of the cellular component of the tumor. After a long quest, the cells of origin of the two forms of this cancer have finally been identified as monoclonal B cells, presenting with clonally rearranged somatically hypermutated immunoglobulin genes. cHL is characterized by the presence of giant multinuclear Hodgkin and Reed-Sternberg (H-RS) cells, whereas lymphocytic and histiocytic (L&H) cells are pathognomonic for nLPHL.

Few insights are available on the molecular pathogenesis of HL. With the exception of chromosomal translocations involving *BCL6* seen in 48% of 23 patients with nLPHL, no other genetic lesions have been reported in nLPHL; moreover, nLPHL cells are negative for EBV. Although H-RS cells in cHL show chromosomal

abnormalities in virtually all cases, H-RS cells carry chromosomal translocations in only a fraction of cases; the oncogenes *BCL-2*, *BCL-6*, *C-MYC*, and *MALT1*, while commonly involved in other types of B-NHL, have been found to be affected only rarely in H-RS cells. In contrast, the NF- κ B pathway is frequently affected in cHL. At the genetic level, somatic mutations of the NF- κ B inhibitor *I κ B α* occur in up to 30% of cases, and a few cases show mutations in another NF- κ B inhibitor, *I κ B ϵ* . Moreover, genomic amplifications of the *REL* gene were reported in 26% of the patients analyzed. EBV infection has been reported in approximately 40% of the patients affected by cHL. Although the precise mechanism linking EBV infection with cHL is unclear, one possibility is the activation of the NF- κ B pathway mediated by the EBV-specific protein LMP1.

Given the abundant reactive tissue surrounding the tumor cells in HL, the microenvironment seems to yield a prominent role in HL. Among the cytokines, chemokines, and receptors that have been shown to have an important role in HL, a prominent role is exerted by IL13 and CD40 and RANK, two members of the tumor necrosis factor (TNF) receptor family.

T-Cell Lymphomas

T-cell lymphomas represent a vastly heterogeneous group of disease (22). Overall they are much less frequent in the population than B-cell lymphomas ($\approx 12\%$) and have been less studied. The clonality of these tumors has been demonstrated through the analysis of the TCR rearrangements. Although these tumors often present with highly rearranged karyotypes, few hallmark translocations have been identified, and little is known about the molecular mechanisms driving tumorigenesis. Adult T-cell leukemia/lymphoma is caused by the HTLV-1 virus. T-cell anaplastic large-cell lymphoma has a recurrent (70%–80% of the patients) translocation, t(2;5), that leads to a fusion protein between *NPM* (nucleophosmin) and *ALK*, a tyrosine kinase receptor belonging to the insulin receptor family not normally expressed in lymphoid cells. *NPM-ALK* reduces apoptosis and increases proliferation through the activation of PI3K/AKT and the RAS pathways.

Multiple Myeloma

Multiple myeloma (MM) is characterized by clonal proliferation of plasma cells in the bone marrow, usually with elevated serum and urine monoclonal paraprotein and associated end-organ sequelae (23). MM is typically preceded by monoclonal gammopathy of undetermined significance (MGUS), a condition present in 1% of adults older than 25 years that reaches 10% incidence in the tenth decade and progresses to MM at a rate of 0.5% to 3% per year. MM is distinguished from MGUS by the number of plasma cells in the bone marrow ($>10\%$) and the presence of osteolytic bone lesions. Two distinct subgroups are present within the population of MGUS/ MM patients, leading to genetically distinct, hyperdiploid and nonhyperdiploid diseases. Hyperdiploid MM involves multiple trisomies of chromosomes 3, 5, 7, 9, 11, 15,

19, and 21, whereas the nonhyperdiploid group more frequently shows mutually exclusive chromosomal translocations (see following sections). Patients presenting with hyperdiploid MM have a better prognosis than those in the hypodiploid group, although a subset of patients within the hyperdiploid group with losses on chromosome 13 and gains on chromosome 1q have a prognosis comparable with the hypodiploid group (24). The translocations characteristic of the hypodiploid group are defined primary, because they are present early in MGUS. In these translocations, the juxtaposition of the enhancer for the IgH locus with genes located in the partner chromosomes dysregulates their expression. The genes that are most frequently involved include gene cyclin D1 (11q13), cyclin D3 (4p16), *MAF* (16q23), or *MAFB* (20q11), resulting in deregulated expression of these target genes in neoplastic plasma cells. In the translocation t(4;14), *FGFR3* and *MMSET* genes at the breakpoint on chromosome 4 are both dysregulated by the genomic rearrangement and are juxtaposed to two different IgH enhancers in the two different chromosomal derivatives. Such translocations, present in MGUS, appear central to MM genesis, whereas progression to MM is associated with mutational activation of *NRAS* or *KRAS* oncogenes in 40% of the cases or alternatively, with *FGFR3* mutations. Late mutational events involve inactivation of *TP53* and secondary translocations involving *MYC* and other genes through mechanisms that do not involve the Ig locus. Despite the identification of several genes located at the translocations and the pervasive cyclin dysregulation in patients with MM, the detailed oncogenic mechanism inducing tumor is poorly understood, with the possible exception of the dysregulation of *MAF* in t(6;14).

The microenvironment exerts a critical role in MM (25). MM plasma cells, except at the most advanced stages of the disease when the cells acquire independence, rely heavily on the interaction with the microenvironment and on the cytokines produced by stromal cells, osteoblasts, osteoclasts, vascular endothelial cells, and lymphocytes. In particular, adhesion molecules like syndecan 1, ICAM1 and NCAM1, and cytokines such as VEGF, IL6, TNF- α , IGF1, and CD40 ligand, have firmly established roles in promoting and supporting the proliferation and survival of MM plasma cells.

Future Directions

Despite the growing body of knowledge that has been accumulating in the past decades on the genetic pathogenesis of hematologic cancers, it is remarkable and in some ways disconcerting how little we know about the pathogenesis and the evolutionary events driving this group of cancers. Given the relevance of this knowledge for the understanding of these diseases and, more pressingly, for the identification of effective therapies, one major challenge facing the scientific community is the full elucidation of the expression programs dysregulated by chromosomal translocations affecting transcription factors. Major breakthroughs have been reached in this field, but few validation studies have been performed on the identified genes and pathways. The second challenge emerging

from initial comprehensive studies using novel technologies such as spectral karyotyping and array comparative genomic hybridizations (CGHs) is the unanticipated level of genomic rearrangements present in these diseases. Although it is likely that at least a subset of such lesions are bystander outcomes of ongoing genomic instability, preliminary results suggest that several lesions affect

genes and pathways especially critical for tumor progression. Higher resolution technologies, more sophisticated bioinformatic and biostatistical tools, and most of all a deeper and more comprehensive understanding of the biology and the pathophysiology of cancer will undoubtedly facilitate the rational design of targeted therapies against these diseases.

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Molecular Genetics of ALL

Acute lymphoblastic leukemia (ALL) is a sharply contrasting disease in the pediatric and adult populations. In children, it is both the most common leukemia and the most common malignancy. Childhood ALL has been emblematic of medical progress, with steady improvement over the past 50 years and a current 5-year event-free survival rate of approximately 80% (Figure 27-1; 1). In contrast, in adults, ALL constitutes a minority of the leukemias and a tiny fraction of all adult malignancies. The long-term disease-free survival for adults with ALL is poor, in the range of 30% to 40% (2). An understanding of molecular genetics is playing an increasingly important role in optimizing therapy in pediatric ALL, defining distinct prognostic subgroups for which therapy can be tailored so that low-risk patients are spared unnecessary toxicity, and high-risk patients receive the intensive therapy most likely to effect a cure. New insights into the molecular biology of ALL in adults may provide the key to improving outcomes for this population in the future.

Epidemiology and Etiology

ALL constitutes 80% of childhood leukemia and 20% of adult leukemia. It has a bimodal distribution, with a first peak between 2 and 5 years of age with an incidence of four to five per 100,000, and a second steady increase beginning at approximately 50 years of age, with a peak incidence of about two per 100,000. A small minority of ALL occurs in patients with known predisposing genetic conditions, which include certain DNA repair defect syndromes, congenital immunodeficiency syndromes, and Down syndrome (3). There is a higher incidence of ALL in twins and first-degree relatives, confirming the contribution of genetic inheritance in sporadic cases as well. However, even in monozygotic twins, concordance is approximately 25%, indicating that environmental factors maintain a significant role. Environmental exposures linked to ALL include ionizing radiation and certain chemotherapeutic agents (although these are far more often associated with development of acute myelogenous leukemia [AML]). Most other proposed environmental associations are controversial or relatively modest. Infectious agents have been causally linked to ALL in a few specific cases: HTLV-1 in adult T ALL/lymphoma, and Epstein-Barr virus (EBV) in mature B-cell ALL/Burkitt lymphoma. An elegant body of research suggests an *in utero* origin

for many cases of childhood ALL (4). Greaves has hypothesized that protection from early childhood infections may cause a pathologic immune response to a later infection that provides selective advantage to the preleukemic clone of fetal origin, and thus precipitates ALL (5).

Prognostic Factors

The cornerstone of ALL therapy is stratification of patients into different risk groups based on a combination of clinical, laboratory, and molecular features, so that the type and intensity of therapy may be tailored appropriately (1). Details of clinical presentation and morphologic and immunophenotypic classification can be found in reviews (1,2) and reference texts (3,6). Age and initial white blood count (WBC) are two powerful and independent parameters used to guide initial therapy. In children, high-risk ALL is defined by the Rome–National Cancer Institute (NCI) consensus criteria as age younger than 1 year or older than 10 years and/or initial WBC count above 50,000/ μL (7). In adults, older age and higher WBC are associated with increasing risk, although there are no standard numeric guidelines. Immunophenotype also plays an important prognostic role. At the molecular level, a wealth of additional information can be gleaned about disease etiology, natural history, clinical prognosis, and therapeutic possibilities.

Overview of Molecular Genetics of ALL

Recurrent abnormalities in ALL can be divided into abnormalities of chromosome number (ploidy) and abnormalities of chromosome structure. Study of recurrent abnormalities has provided much information about underlying leukemogenic mechanisms and clinical prognosis. Chromosomal translocations are a frequent type of structural abnormality in ALL. Translocations generally cause two types of events. A proto-oncogene may be brought into proximity with a T-cell receptor (TCR) or immunoglobulin locus, causing its overexpression, or the genes at the breakpoints of the rearranged chromosomes, often transcription factors, may fuse to form a new, chimeric protein that is oncogenic due to altered properties and/or expression patterns (8). Specific abnormalities are discussed in the following sections and summarized in Table 27-1.

FIGURE 27-1 Kaplan-Meier analysis of overall survival in 2,628 children with newly diagnosed acute lymphocytic leukemia (ALL). The patients participated in 15 consecutive studies conducted at St. Jude Children's Research Hospital from 1962 to 2005. The 5-year overall survival estimates ($\pm$ SE) are shown, except for Study 15, for which preliminary results at 4 years are provided. (From Pui CH, Evans WE. *Treatment of acute lymphoblastic leukemia*. *N Engl J Med* 2006;354:166–178, with permission.)

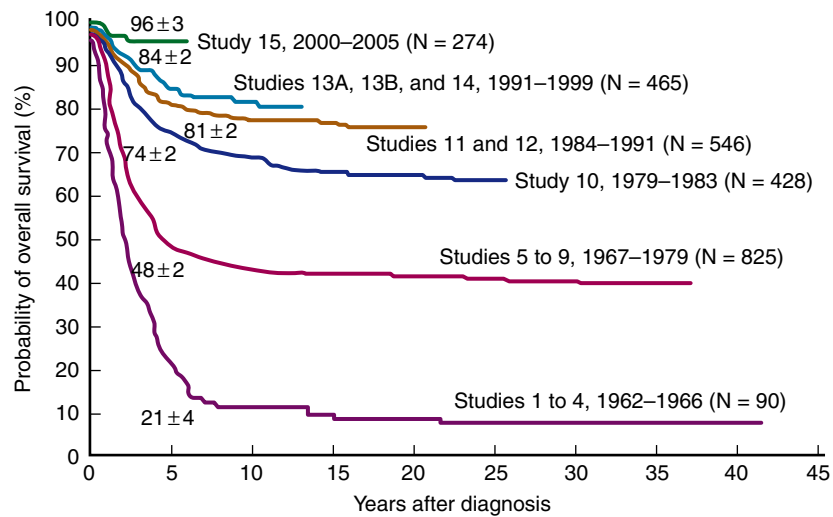


Table 27-1 Selected Genetic Abnormalities Associated with ALL

Chromosomal Abnormality	Genes Involved	Pediatric (%)	Adult (%)	Mechanism of Transformation	Prognostic Impact ^a
Pre-B ALL					
t(12;21)(p13;q22)	TEL-AML1	25	2	Represses AML1 function as transcriptional activator	Favorable
t(1;19)(q23;p13)	E2A-PBX1	6	3	Promotes PBX1 function as transcriptional activator	Formerly poor; negated by intensive therapy
t(17;19)(q23;p13)	E2A-HLF	<1	<1	Repression of E2A and anti-apoptotic effects (?)	Poor
t(9;22)(q34;q11)	BCR-ABL	3	25	Increased tyrosine kinase activity	Poor
t(4;11)(q21;q23)	MLL-AF4	8	10	Disruption of HOX expression patterns	Poor
T ALL					
t(1;14)(p34;q11)	TAL1, TC- α/β	7	12	Repression of E2A transcriptional activity	Poor vs. no prognostic significance
t(11;14)(p15;q11)	LMO1, TCR α/δ	<1	<1	LMO1 activation; repression of E2A transcriptional activity	Unknown
t(11;14)(p13;q11)	LMO2, TCR α/δ	1	<1	LMO2 activation; repression of E2A transcriptional activity	Unknown
t(10;14)(q24;q11); t(7;10)(q35;q24)	HOX11, TCR δ or TCR β	0.7	8	Dysregulated expression of intact HOX11	Favorable if intensive therapy
t(5;14)(q35;q32); t(5;14)(q35;q11)	HOX11L2, BCL11B or TCR δ	2.5	1	HOX11L2 activation	Poor vs. no prognostic significance
t(8;14)(q24;q11)	MYC, TCR	<1	<1	MYC overexpression	Unknown
t(7;19)(q35;p13)	LYL1, TCR β	1.5	2.5	LYL1 overexpression	Unknown
Mature B ALL					
t(8;14)(q24;q32)	MYC, IgH	2	4	MYC overexpression	
t(8;22)(q24;q11)	MYC, Ig λ	<1	<1	MYC overexpression	
t(2;8)(p12;q24)	Ig κ , MYC	<1	<1	MYC overexpression	

ALL, acute lymphoblastic leukemia.
^aSee text for details.

Cytogenetic analysis using karyotype and fluorescent *in-situ* hybridization (FISH) are the current standard methods used for clinical diagnostic evaluation. Microarray-based analysis of gene expression patterns is another powerful tool that has been applied increasingly over the past few years to provide biological validation of existing ALL subtypes and identify new ones; to discriminate, within existing subgroups, between good and poor outcomes; and to identify highly overexpressed genes that are candidates for development of new targeted therapies (9,10).

Epigenetics, the study of heritable changes in gene function that occur without a change in DNA sequence, has been recognized as an alternative path to malignancy (11). CpG island methylation has been reported as an independent poor prognostic factor in T- and B-lineage ALL (12,13).

Abnormalities of Chromosome Number (Ploidy)

Ploidy can be assessed by chromosome number or flow cytometry using the DNA index (DI), the ratio of fluorescence in leukemic blasts compared with normal cells. Normal diploid cells have 46 chromosomes and a DI of 1.0, hyperdiploid cells have higher values, and hypodiploid cells have lower values. Hyperdiploidy is further classified as “low” (47–50) and “high” (>50).

Hypodiploid cases constitute approximately 6% of pediatric and 2% to 8% of adult ALL (14). Only those with fewer than 45 chromosomes have significantly worse outcome, with the worst outcome in near-haploid cases (24–28 chromosomes; 14). The adverse prognostic impact in adults is somewhat weaker (15). “Pseudodiploid” cases, with normal chromosome number but structural abnormalities, also do relatively poorly. Rare cases with

near triploidy (68–80) or near tetraploidy (>80) have also been associated with very poor outcome.

Hyperdiploidy occurs in about 35% of pediatric and 25% of adult ALL (15,16). It usually coincides with other favorable risk factors, but retains independent positive predictive value in children; its prognostic impact in adults is less clear. Independent favorable prognostic impact may be attributable specifically to trisomies of chromosomes 4, 10, and 17, rather than to total chromosome number (Figure 27-2; 17). The biologic basis for hyperdiploidy ALL remains poorly defined. *RAS* and *FLT3* mutations and *FHIT* hypermethylation occur more frequently in this subtype.

Abnormalities of Chromosome Structure

TEL-AML1, t(12;21)(p13;q22)

The TEL-AML1 fusion protein formed by the t(12;21)(p13;q22) translocation is the most frequent abnormality in children, occurring in approximately 25%. It is much rarer in adults, constituting only 2% (18). TEL (translocation-ETS-leukemia) is also known as *ETV6* (ETS variant gene 6). AML1 is also known as CBFA2 (core binding factor A2) and most recently, *RUNX1* (*runt*-related transcription factor 1). In nearly all cases the translocation is cryptic, involving a region too small to be detected by karyotype.

TEL encodes a widely expressed nuclear protein belonging to the Ets family of transcription factors, which are involved in diverse developmental processes including establishment of embryonic and adult hematopoiesis. A helix-loop-helix (HLH) region known as the ETS domain allows DNA binding for transcriptional regulation, and an HLH region known as the pointed domain appears to facilitate self-association (Figure 27-3A). AML1 is a transcription factor with

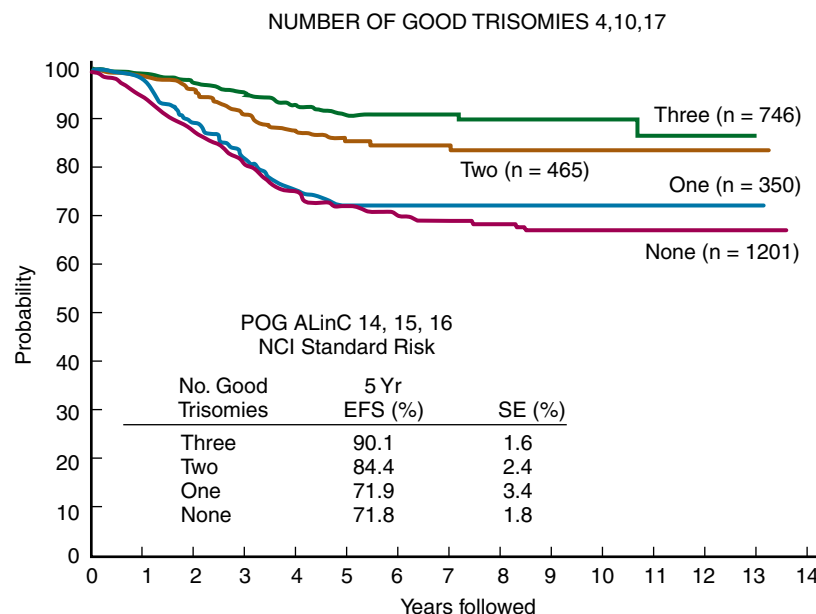


FIGURE 27-2 Kaplan-Meier analysis of event-free survival relative to number of good trisomies 4, 10, and 17. Data are for children with B-precursor National Cancer Institute standard-risk acute lymphocytic leukemia enrolled on Pediatric Oncology Group (POG) protocols ALinC 14, 15, 16 series. Similar data (not shown) were obtained for children enrolled on Children's Cancer Group (CCG) protocols 1800s/1922 series. (From Sutcliffe MJ, Shuster JJ, Sather HN, et al. High concordance from independent studies by the Children's Cancer Group (CCG) and Pediatric Oncology Group (POG) associating favorable prognosis with combined trisomies 4, 10, and 17 in children with NCI Standard-Risk B-precursor Acute Lymphoblastic Leukemia: a Children's Oncology Group (COG) initiative. *Leukemia* 2005;19:734–740, with permission.)

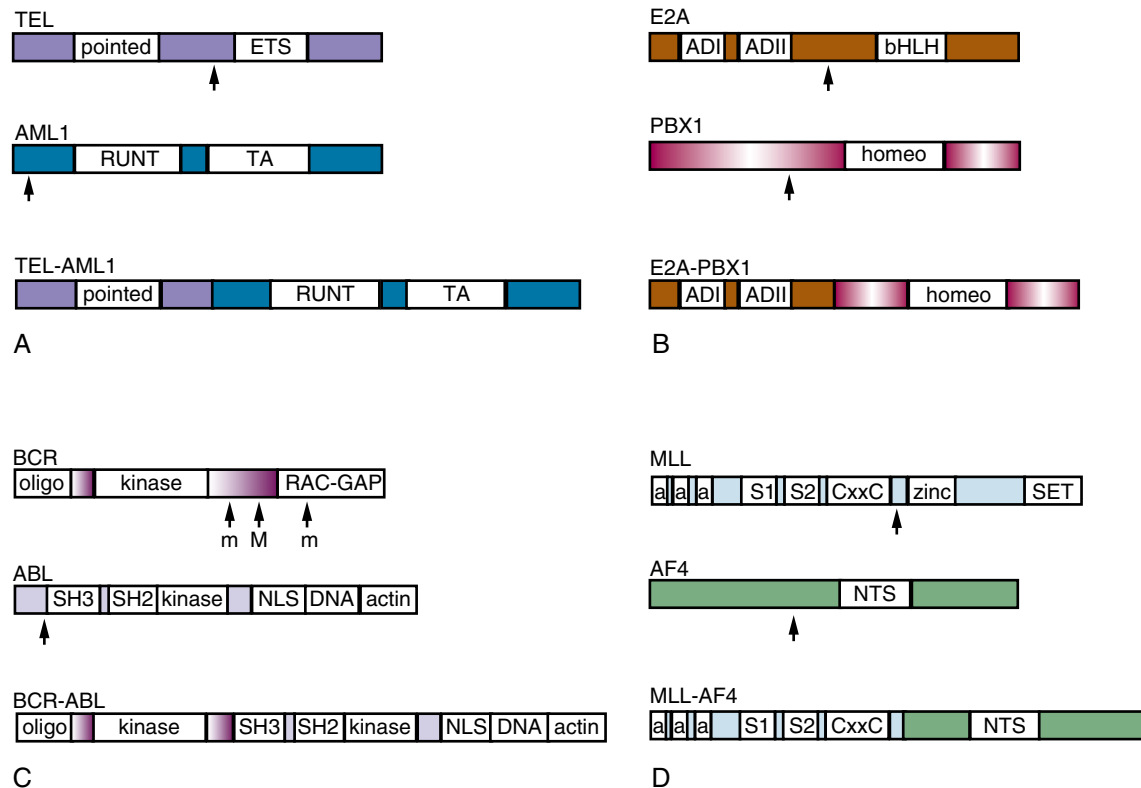


FIGURE 27-3 SCHEMATIC OF KEY DOMAINS OF THE GENES INVOLVED IN SEVERAL PRINCIPAL TRANSLOCATIONS IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) AND THE TRANSLOCATION PRODUCTS. ARROWS INDICATE COMMON BREAKPOINTS. NOTE THAT LOSS OF THE DOMAIN CONFERRING SEQUENCE-SPECIFICITY TO TRANSCRIPTION FACTOR BINDING OCCURS IN TEL-AML1 (ETS DOMAIN OF TEL) AND E2A-PBX1 (bHLH DOMAIN OF E2A). GENE REGIONS ARE NOT DRAWN TO SCALE. **A:** TEL-AML1; TA, transactivation domain. **B:** E2A-PBX1. ADI and ADII, activation domains I and II; bHLH, basic helix-loop-helix, sequence-specific DNA-binding domain; Homeo, homeobox domain. **C:** BCR-ABL. All three breakpoint regions are indicated (M, major, for p210 protein; m, minor, for p190 protein; μ , associated with p230 protein). Only the p190 fusion product is illustrated. Oligo, oligomerization domain; kinase, serine-threonine kinase domain; RAC-GAP, RAS-like GTPase; SH3, SH2, and kinase, SRC homology domains; kinase, tyrosine kinase domain; NLS, nuclear localization domains; DNA, DNA-binding site; actin, G and F actin binding site. **D:** Mixed lineage leukemia. a, AT-hook; CxxC, cysteine-rich motif homologous to DNA methyltransferase; S1 and S2, subnuclear localization domains; NTS, nuclear targeting sequence; zinc, zinc fingers.

highly restricted expression in hematopoietic cells and developing ganglions. AML1 binding to an enhancer core sequence is required for transcription of several hematopoietic-specific genes, and may organize the factor complex necessary for lineage-specific transcription. AML1's affinity for the enhancer core motif is enhanced by heterodimerization with core-binding factor β (CBF- β). Like TEL, AML1 is critical for establishment of hematopoiesis of all lineages.

The TEL-AML1 fusion protein is widely expressed due to the TEL promoter and converts AML1 from a transcriptional activator to a repressor. The exact mechanism of repression is unclear, as is the manner in which this mediates leukemogenesis. TEL-AML1 overexpression causes leukemia in only a minority of mouse models, with low penetrance and prolonged latency, suggesting that additional events are crucial for full transformation. A frequent secondary event in TEL-AML1⁺ ALL is loss-of-heterozygosity (LOH), deletion or otherwise down-regulated expression of the remaining normal copy of TEL, suggesting a potential role for TEL as a tumor suppressor.

TEL-AML1 positivity tends to occur in children 1 to 10 years old and nearly exclusively in CD10⁺ B-precursor ALL. TEL-AML1⁺ ALL has a hallmark tendency to relapse late, with excellent chemosensitivity and salvage rate. Relapse may in fact represent evolution of a new leukemic clone from the preleukemic TEL-AML1⁺ cell of origin. On intensive treatment regimens,

TEL-AML1 is associated with superior prognosis, though it may not retain prognostic significance independent of age and presenting WBC in the modern treatment era (19).

E2A-PBX1, t(1;19)(q23;p13)

The E2A-PBX1 fusion protein, associated with the t(1;19)(q23;p13) translocation, is the second most common translocation in pediatric ALL, occurring in approximately 6% of all pre-B-cell ALL (20). It is a rare (3%) and adverse feature in adults (21). The fusion protein combines the two activation domains of the basic helix-loop-helix (bHLH) transcription factor E2A on chromosome 19 with the homeobox (HOX) gene PBX1 (for pre-B-cell homeobox 1) on chromosome 1, resulting in a strong transcriptional activator effect on PBX1 (Figure 27-3B). E2A is a transcriptional activator critical in lymphocyte development, as well as widely expressed and influential in diverse cellular processes. PBX1 belongs to the TALE (three-amino-acid loop extension) class of atypical homeodomain proteins. The homeodomain mediates DNA binding and HOX gene interaction.

The E2A-PBX1 chimeric transcription factor strongly activates a subset of HOX genes normally regulated by PBX1. The basis for its transforming ability may be reduction of wild-type E2A levels, aberrant activation of PBX1 targets in pre-B cells, or

activation of targets not normally regulated by *PBX1*, which are affected by the E2A-PBX1 fusion protein (22,23). Fusion protein overexpression in mouse models causes a variety of leukemias, although not B-lineage ALL, suggesting potent nonlineage-specific transforming activity. Unlike other ALL translocations, t(1;19)+ ALL does not show evidence of *in utero* origin.

E2A-PBX1 positivity often coincides with other high-risk factors. Early studies showed independent adverse prognostic impact, but on modern intensive regimens, survival is equivalent (20,23,24). The t(1;19) occurs most often as an unbalanced translocation. Cases with a balanced translocation have demonstrated poorer survival in some studies but not others.

E2A-HLF, t(17;19)(q23;p13)

The t(17;19) translocation is a much rarer event involving E2A, occurring in approximately 1% of pediatric ALL and rarely in adults (25). The HLF (for hepatic leukemia factor) fusion partner is a transcription factor not normally expressed in hematopoietic cells. Potential oncogenic effects of the fusion protein include repression of normal E2A function, altered transcriptional activity of HLF, and promotion of lymphoblast survival possibly via anti-apoptotic effects. E2A-HLF ALL tends to occur in adolescents and is frequently associated with hypercalcemia; disseminated intravascular coagulopathy (DIC); cIgM-negative, low CD10 positivity pro-B-cell immunophenotype; and a poor prognosis despite intensive chemotherapy (23).

BCR-ABL, t(9;22)(q34;q11)

The t(9;22) translocation was the first recurrent chromosomal abnormality identified in human cancer, in association with chronic myelocytic leukemia (CML; 26). This translocation, known as the Philadelphia chromosome (Ph), is an essential criterion in the diagnosis of CML and the most frequent abnormality in adult ALL, occurring in 25% of adult ALL with a frequency that increases markedly with age, and in only about 3% of pediatric cases (18,27). Ph occurs primarily in CD10⁺ precursor-B ALL, with frequent coexpression of myeloid markers. Ph is a significant adverse prognostic marker, with significantly lower induction rates, more frequent and earlier relapse, and poorer overall survival (28).

The Philadelphia chromosome is formed by in-frame fusion of the 5' portion of *BCR* (for breakpoint cluster region) on chromosome 22 to the 3' portion of the tyrosine kinase *C-ABL* on chromosome 9, a proto-oncogene that is part of the RAS signaling pathway (Figure 27-3C). The fusion protein up-regulates ABL tyrosine kinase activity. Two main fusion proteins occur, which differ in BCR breakpoint. Breaks within the 5.8-kb major breakpoint cluster region (M-BCR), occurring in CML and 25% of adult Ph⁺ ALL, form a 210-kD protein known as p210. In the remainder of adult ALL and most pediatric ALL, the breakpoint occurs further upstream, in the minor breakpoint cluster region (m-BCR), forming a 185- to 190-kD protein usually known as p190. An additional breakpoint generates a 230-kD protein associated with a rare CML variant with neutrophilia, and occasionally with classic CML. It has been suggested that the p190 protein arises *de novo*, whereas the p210 protein may represent the blast

crisis of a previously unrecognized CML. Other features that may distinguish cases of Ph⁺ ALL that originated as CML include basophilia, marked splenomegaly, and persistence of the BCR-ABL fusion protein in hematopoietic precursor cells of all lineages following remission.

ABL is a nonreceptor tyrosine kinase with multiple complex functions that is predominantly nuclear and ubiquitously expressed, but normally under tight regulation. BCR is a ubiquitously expressed signaling protein of unclear significance both in normal biology and as part of the BCR-ABL fusion protein. Properties of the fusion protein that appear to be crucial for transformation include translocation to the cytoplasm, constitutive elevated tyrosine kinase activity, oligomerization, autophosphorylation, actin-binding, DNA-binding; and interaction with the RAS and BCL2 signaling pathways. All three isoforms (p190, p210, and p230) encode active tyrosine kinases, transform cell lines, and cause leukemia in transgenic mice (29,30). Both the p210 and particularly the p190 fusion transcripts are detectable in remission using sensitive polymerase chain reaction (PCR) methods as well as in many healthy individuals, suggesting that despite the importance of the BCR-ABL fusion protein in leukemic transformation, additional steps are required as well.

Patients with Ph⁺ ALL tend to be older, with higher leukocyte and peripheral blast count, central nervous system (CNS) involvement, lower induction rates, shorter remission duration, and poorer overall survival. In both adults and children, allogeneic stem cell transplant has generally been regarded as the only curative therapy and is generally recommended in first complete remission (CR). If a related donor is unavailable, an unrelated donor may be considered. Relatively favorable features include initial WBC below 25,000/ μ L; good initial response to prednisone, and possibly the p190 protein compared with the p210.

Monosomy 7 and/or loss of 9p are secondary aberrations that may be associated with worse outcome. Gain of more than one Ph chromosome is associated with progression to blast crisis and poor outcome in CML, but the significance in Ph⁺ ALL is less clear. Presence of the BCR-ABL fusion transcript indicates a negative prognosis even if the Ph chromosome is undetectable, highlighting the utility of PCR compared with karyotype. Molecular (PCR-negative) remission is significantly more likely to be durable than cytogenetic remission with molecular positivity, both before and after transplant.

Treatment of CML and Ph⁺ ALL was revolutionized by imatinib mesylate, also known as STI-571 or Gleevec, in 2001 (31). Imatinib, a selective tyrosine kinase inhibitor, was the first molecularly targeted therapy to attain large-scale clinical success, fulfilling the goals of antitumor selectivity and low systemic toxicity. Despite its success, it has not been effective as a single agent due to the rapid development of resistance, and allogeneic stem cell transplant in first CR remains the optimal curative therapy. However, imatinib has improved CR rates in adults before transplant, and combined with low-dose interferon has produced prolonged remission in patients who are not transplant candidates. In pediatrics, early-phase trials of imatinib have been conducted, and it has been integrated into front-line regimens. New developments in the treatment of Ph⁺ ALL include more potent second-generation tyrosine kinase inhibitors, dual SRC and BCR-ABL kinase inhibitors, and combination therapy with a farnesyl transferase or PI3K inhibitor.

MLL, 11q23 Rearrangements

MLL gene rearrangements occur in 8% of pediatric ALL and 10% of adult ALL (18) and constitute the most frequent abnormality in infant ALL, occurring in 60% to 70% of cases (32). They are also associated with AML, particularly secondary malignancies following anthracyclines and epipodophyllotoxins. MLL-rearranged leukemias are unusual in two respects: (1) the N-terminal of MLL forms a fusion protein with the C-terminal of over 40 different partners, including itself, and (2) MLL rearrangements are found in both ALL and AML, whereas most other translocations are lineage specific. Indeed, MLL takes its name, “mixed lineage leukemia,” from this distinguishing feature (it is also known as HRX or HTRX for homology to *trithorax* in *Drosophila*; and ALL1 for involvement in ALL). MLL-rearranged ALL has a unique gene expression pattern suggestive of arrest at an earlier hematopoietic progenitor stage than other ALL subgroups (10). The MLL-AF4 fusion protein formed by t(4;11) is the most common MLL translocation in ALL, making up 70% of cases. The MLL-ENL fusion formed by t(11;19) comprises another 13%.

Infant ALL with MLL rearrangement tends to be associated with age less than 6 months, female gender, massive tumor burden, organomegaly, frequent CNS involvement, coexpression of myeloid antigens, and CD10-negative pro-B immunophenotype. MLL rearrangement is a poor prognostic feature in infant ALL and to a lesser extent in children older than 1 year (33). One surprising exception is MLL-ENL, associated with good prognosis in both T ALL and patients 1 to 9 years old (34,35).

MLL is a large protein consisting of multiple motifs, including AT-hook DNA-binding domains, transcriptional activation and repression zinc finger domains, and a highly conserved SET domain that regulates homeotic (*Hox*) promoters. Several motifs are homologous to the *Drosophila trithorax* protein, which maintains appropriate expression of the *Hox* genes controlling segment determination. In normal hematopoiesis, MLL is required to generate stem cell progenitors and to establish lymphoid and myeloid lineages. In general, the many MLL fusion partners fall into the broad categories of signaling molecules and nuclear transcription factors (36). The most frequent MLL partner, AF4, is a ubiquitously expressed gene necessary for normal hematopoiesis.

Since *Hox* genes can induce leukemia, it is thought that MLL rearrangements mediate leukemogenesis through disruption of normal *Hox* expression patterns (36,37). The contributions of MLL's many fusion partners to leukemic transformation remain unclear, since they do not all share structural or functional similarities (Figure 27-3D). One common function may be converting MLL to a constitutive transcriptional effector. It is unclear whether loss-of-function and/or fusion partner protein functions play a significant role as well.

Both infant and treatment-related leukemias show nonrandom clustering of MLL breakpoints within exons distinct from the breakpoints in *de novo* MLL-rearranged leukemias, suggesting that they share a common mechanism of leukemogenesis (38). One hypothesis is that infant ALL arises from *in utero* exposure to topoisomerase II inhibitors, analogous to the initiating insult in therapy-related MLL-rearranged leukemias (39). Epidemiologic

studies of the association between infant leukemia and *in utero* exposure to topoisomerase II inhibitors have identified a modest association between dietary intake and MLL-rearranged AML, and a stronger association between certain medications and insecticides and MLL-rearranged ALL (40,41).

Event-free survival in infant ALL is generally poor, ranging from 20% to 40%, and in cases with t(4;11) it is particularly dismal. Induction rates are generally comparable to other ALL, but early relapse is frequent, usually within a year of diagnosis. MLL-rearranged ALL shows relative resistance *in vitro* to glucocorticoids and L-asparaginase, and sensitivity to cladribine and cytarabine (42). High-dose cytarabine has been incorporated into infant ALL regimens with modest success. The receptor tyrosine kinase FLT3 is highly expressed in MLL-rearranged ALL (10,43), and the use of FLT3 inhibitors and other novel molecularly targeted therapies is under investigation (44).

T ALL

T ALL makes up 12% of pediatric and 25% of adult ALL (18). It occurs most often in adolescents and young adults, frequently presenting with an extremely high WBC, CNS involvement, a mediastinal mass, marked lymphadenopathy, and hepatosplenomegaly. Human T-cell leukemia virus (HTLV) I and II infection are predisposing factors in a subset of adult T ALL. Historically, survival was dismal compared with that of B-lineage ALL, but with intensified therapies it has improved to approximately 70% to 75% in pediatrics (45) and 45% in adults (46). However, traditional risk factors such as age and WBC used for stratification in B-lineage ALL appear not to be as prognostically informative in T ALL, highlighting the importance of identifying molecularly based prognostic differences instead (45,47,48).

The leukemogenic event in T ALL typically involves overexpression of an unaltered proto-oncogene, rather than generation of a novel fusion protein, due to a translocation placing the proto-oncogene under control of a TCR promoter or enhancer, most often TCR- β or TCR- α/δ . The breakpoints occur at junctions where RAG recombinase acts during normal V(D)J recombination, suggesting they are the consequence of physiologic gene rearrangement processes gone awry (49).

Numerous transcription factors have been identified as aberrantly expressed through juxtaposition to TCR loci in T ALL. They include MYC and several homologous basic helix-loop-helix (bHLH) proteins: TAL1, TAL2, LYL1, and BHLHB1. Homeobox genes are another important target of dysregulation in T ALL: the entire HOXA cluster, HOXA10, HOXA11, HOX11, HOX11L2, LMO1, and LMO2. Oncogenic fusion proteins are less common but do occur, such as MLL-ENL; CALM-AF10; rarely ETV6-JAK2; and three ABL1 fusions: ETV6-ABL1, EML1-ABL1, and NUP214-ABL1. The latter exemplifies a novel mechanism for activation of tyrosine kinases in cancer: formation of episomes or extrachromosomal genetic material (50). The cryptic fusion results in ABL1 activation, and preliminarily these leukemias appear susceptible to the tyrosine kinase inhibitor imatinib. Another class of mutations causes overexpression of components of the TCR-signaling pathway such as SRC-family tyrosine kinase, LCK, and NRAS.

Aberrant TAL1 expression via a variety of mechanisms occurs in over 60% of T ALL. The oncogenic activity of TAL1 seems to occur through sequestration and inactivation of E2A within a binding complex. LMO1 and LMO2 are transcription factors that play important roles in hematopoiesis and vascular development, serving as an interface for binding of multiple transcription factors in a large complex. TAL1 forms a heterodimeric DNA-binding complex with either LMO1 or LMO2, which cooperates in leukemogenesis at least in part through inhibition of E2A.

A disturbing confirmation of the oncogenicity of LMO2 occurred during a gene therapy trial for X-linked SCID caused by γ -chain deficiency (51,52). Patients underwent retrovirus-mediated γ -chain gene transfer into autologous bone marrow progenitor cells. Approximately three years later, two of the ten patients developed T ALL. The apparent mechanism was retroviral insertional mutagenesis. In both cases, the retroviral particle integrated close to the LMO2 locus, and presumably exerted enhancer activity on the LMO2 promoter causing its overexpression in lymphoblasts.

The heterogeneous molecular genetics of T ALL was partially unified in 2004 with the discovery of a single gene up-regulated in over 50% of cases (Figure 27-4A and 27-4B; 53). The gene is *NOTCH1*, a regulatory transmembrane receptor that plays a crucial developmental role in cell fate determination and pattern formation, hematopoietic stem cell maintenance, and T cell fate specification in the mature organism (54). *NOTCH1* was first identified in the rare T ALL translocation $t(7;9)(q34;q34.3)$, which juxtaposes it to the TCR- β locus, leading to overexpression of a constitutively activated, truncated protein. More significantly, somatic activating *NOTCH1* mutations occur in over 50% of human T ALL cases (53).

Poor prognostic features in T ALL include CD10 positivity, pro-T immunophenotype, and, possibly, TAL1 expression. *HOX11* overexpression is generally associated with a favorable outcome, at least with modern intensive therapy. *HOX11L2* overexpression was reported in some studies as a poor prognostic feature, but intensive therapy appears to eliminate this effect. Cases with the NUP214-ABL1 fusion appear aggressive and may benefit from imatinib. MRD positivity is a significant adverse prognostic marker in T ALL, occurring more often and correlating more closely with relapse than B-lineage ALL. Research data suggest that *NOTCH1* mutations may be associated with a favorable prognosis (Figure 27-4C; (55)).

Compound 506U, also known as nelarabine, is a nucleoside analogue preferentially accumulated in T ALL that is being incorporated in current clinical trials (56). Since mutated *NOTCH1* activity depends on γ -secretase activity, γ -secretase inhibitors are also being investigated for therapeutic use.

c-MYC, $t(8;14)(q24;q32)$

One of the first links between a chromosomal translocation, oncogene overexpression, and development of a human cancer occurred in the early 1980s with the discovery of c-MYC

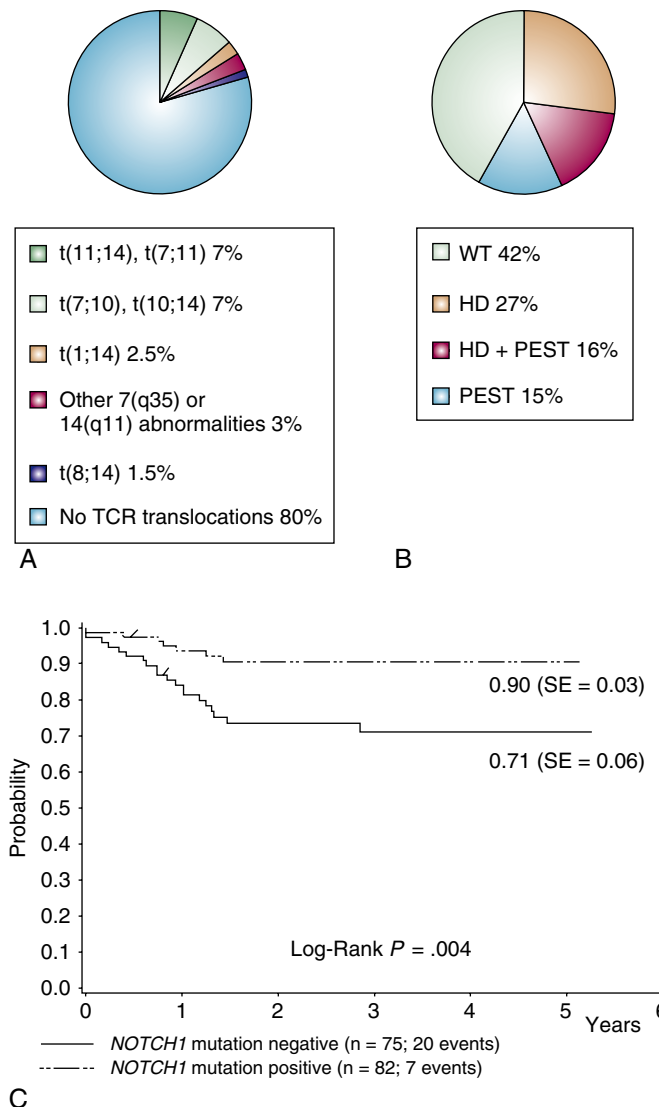


FIGURE 27-4 Distribution of common translocations and *NOTCH1* mutations in T-cell acute lymphocytic leukemia (T ALL) and effect of *NOTCH1* mutation status on outcome. **A:** Distribution of the most common translocations involving the TCR loci among 201 cases of pediatric T ALL. **B:** Distribution of *NOTCH1* mutations in 187 cases of pediatric T ALL. Note that only 42% of cases do not show these mutations. *NOTCH1* mutations may affect the heterodimerization domain (HD), the PEST (polypeptide enriched in proline, glutamate, serine, and threonine) destruction box, or both. WT = wild type. **C:** Kaplan-Meier analysis of event-free survival at 4 years in children with T ALL with or without *NOTCH1* mutations treated on the ALL-BFM 2000 study. (**A, B:** From Grabher C, von BH, Look AT. *Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia*. *Nat Rev Cancer* 2006;6:347–359; **C:** from Breit S, Stanulla M, Flohr T, et al. *Activating NOTCH1 mutations predict favorable early treatment response and long-term outcome in childhood precursor T-cell lymphoblastic leukemia*. *Blood* 2006;108:1151–1157, with permission.)

dysregulation in Burkitt lymphoma (57,58). The $t(8;14)(q24;q32)$ translocation places c-MYC on chromosome 8 under control of the immunoglobulin heavy-chain gene on chromosome 14, resulting in constitutive c-MYC overexpression. MYC dysregulation is also an essential feature of mature B-cell leukemia, also known as Burkitt leukemia. In essence, Burkitt lymphoma and mature B-cell leukemia are best viewed as two manifestations of a common disease, differing only in extent of dissemination. The similarity of their symptomatology, prognosis, and treatment illustrates

the importance of a common underlying genetic mechanism in defining disease biology.

Over 90% of mature B-cell ALL exhibits the t(8;14) translocation. In the remainder, t(2;8)(p12;q24) or t(8;22)(q24;q11) place MYC under control of the κ or λ light chains, respectively. Another variant, t(8;14)(q24;q11), involves the TCR- α locus and has been reported in association with T ALL. Outcome for mature B-cell ALL has improved substantially since the shift from standard ALL therapy to much shorter, more dose-intensive regimens.

7p Deletions and Monosomy 7

Losses involving chromosome 7 are more common in AML, but they do occur in approximately 5% of adult and pediatric ALL (59). In adult ALL, deletion or loss of chromosome 7 is often associated with Ph positivity, and does not have independent prognostic significance. In pediatric ALL, chromosome 7 losses tend to occur in patients with other concomitant adverse risk factors, but retain some independent negative prognostic impact.

9p21 Deletion

Deletions in the 9p21–22 region occur in 10% to 30% of ALL cases and over 50% of T ALL, with the incidence rising as cytogenetics laboratories adopt more sensitive detection methods (60). The principal targets appear to be the INK4A and INK4B loci, which contain two cyclin D kinase inhibitors, p16 and p15, which prevent abnormal cells from passing through the G₁ cell cycle checkpoint and are mutated, deleted, or epigenetically silenced in many cancers. The 9p21 deletion often coincides with other adverse factors; its independent significance is controversial.

Cooperating Pathways: *p53*, *FLT3*, *Ras*, and *PTPN11*

P53 is a classic tumor suppressor gene that initiates cell cycle arrest or apoptosis in response to abnormal proliferation, hypoxia, or DNA damage and is mutated in many human cancers. Mutation of *p53* itself is rare in ALL, but mutations of elements of the pathway such as p14^{ARF} and p21^{CIP1} are common and may be associated with an unfavorable prognosis. *FLT3* (for FMS-like tyrosine kinase) is a membrane-bound receptor tyrosine kinase involved in hematopoietic proliferation, differentiation, and apoptosis. Mutations resulting in ligand-independent activation are oncogenic and occur in 15% to 35% of AML cases and 1% to 3% of ALL (61). *FLT3* mutations in ALL are particularly associated with MLL rearrangement and hyperdiploidy. *Ras* family members play crucial signaling roles in proliferation, anti-apoptosis, and other processes. Mutations in *NRAS* and *KRAS* have been linked to parental exposures to certain drugs and hydrocarbons. Activating mutations of *PTPN11*, which encodes the tyrosine phosphatase SHP-2, enhance *Ras* signaling. Forty-five percent of TEL-AML1–negative pre-B ALL have mutations in *PTPN11*,

NRAS, and/or *KRAS2* (62). *RAS* mutations do not appear prognostically significant, although the significance of *PTPN11* mutations remains unclear.

Clinical Implications of Genetic Lesions in ALL

At diagnosis, the main adverse features which affect treatment decisions in pediatric ALL are BCR-ABL, E2A-PBX1, MLL rearrangements, and MYC rearrangements. Favorable features are TEL-AML1 and hyperdiploidy or the presence of particular trisomies such as 4, 10, and 17. In adults, BCR-ABL and MLL rearrangement may affect treatment choices, whereas the other abnormalities are too rare or uncertain in impact to be routinely assessed or acted upon.

Detection of molecular abnormalities in ALL has important implications for disease monitoring as well as initial risk assignment. The classical definition of remission requires fewer than 5% lymphoblasts in the bone marrow by morphologic examination. However, newer molecular definitions of remission based on minimal residual disease (MRD) detection are far more sensitive in identifying patients at risk of ultimate morphologic relapse. MRD can be measured by flow cytometry or PCR and sensitivity ranges from 10^{−3} to 10^{−6}. Numerous pediatric studies have demonstrated that MRD positivity has poor prognostic impact independent of other risk factors, at time points ranging from early induction to 24 months into treatment. Conversely, rapid achievement of MRD negativity before the end of induction identifies a favorable risk group, who might be spared the adverse effects of high-intensity regimens. Adults tend to have higher levels, more frequent, and more prolonged duration of MRD positivity, not surprisingly given the comparative drug resistance of their disease. Nevertheless, MRD does appear to have independent predictive value in the adult population as well.

Conclusion

Unraveling the molecular genetics of ALL has paved the way toward many advances in our understanding of leukemogenesis, our ability to stratify patients by risk group at diagnosis and track disease status during treatment, and identification of novel therapeutic targets. A deepening appreciation of the significance of molecular characteristics of the host, as well as the disease, is beyond the scope of this chapter but has provided complementary information to further individualize the approach to optimizing therapy. Future challenges include optimizing therapy for existing poor-risk disease subtypes, and identifying patients within favorable-risk subtypes who nevertheless fail to be cured and devising more effective therapies for them. On a practical level, it will be an increasing challenge to take the mushrooming quantity of molecular and genomic research on ALL and extract those principles that are most informative and feasible for large-scale application in the clinical realm.

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Biology of Chronic and Acute Myeloid Leukemia

The human acute leukemias are clonal disorders that arise from hematopoietic progenitors developing in the lymphoid or myeloid pathway or from primitive stem cells with multilineage potential. The genesis of these disorders involves the deregulation of the differentiation and maturation programs of the hematopoietic myeloid lineage that originates from primitive stem cells with multilineage potential. Chronic myeloid leukemia (CML) was the first cancer associated with a specific chromosomal abnormality. This aberrancy, first identified as a minute chromosome, is now known as the Philadelphia chromosome, which results from the t(9;22) balanced reciprocal translocation. The molecular consequence of the Philadelphia translocation is the generation of the oncogene BCR-ABL1 that encodes the chimeric BCR-ABL protein with constitutive kinase activity. The other group of myeloid leukemias is collectively grouped under the generic term of “acute myeloid leukemia” (AML), which is clinically characterized by accumulation of immature blastic cells that exhibit uncontrolled proliferation and lack of normal differentiation.

Chronic Myeloid Leukemia

CML results from the neoplastic transformation of a hematopoietic stem cell. The hallmark genetic abnormality driving the pathogenesis of CML is the balanced translocation t(9;22)(q34;q11), which, cytogenetically results in the Philadelphia chromosome(1), and molecularly gives rise to the BCR-ABL1 hybrid gene. The BCR-ABL1 gene encodes a protein kinase, BCR-ABL, which is constitutively activated in CML. Classically, CML has been described as a disease that evolves in three phases. Most patients are diagnosed in chronic phase (CP), characterized by overproduction of immature myeloid cells and mature granulocytes in the bone marrow and peripheral blood. Left untreated, most patients progress to the blastic phase (BP), characterized by a peripheral blood or bone marrow blast percentage of over 30%, usually preceded by an acute phase (AP).

The BCR-ABL1 Oncogene

ABL1 is the human homologue of the *v-abl* oncogene carried by the Abelson murine leukemia virus (A-MuLV). ABL1 encodes the 145-kD nonreceptor tyrosine kinase ABL that localizes at several

subcellular sites, interacting with a large variety of cellular proteins, including signaling adaptors, other kinases, phosphatases, transcription factors, and cytoskeletal proteins(2). In turn, BCR encodes a protein with serine-threonine kinase activity. The fusion of the BCR and ABL1 genes results in the activation of the c-ABL1 proto-oncogene to its oncogenic form. Several experimental models have demonstrated that the BCR-ABL1 is central to the pathogenesis of CML. This provides the rationale for the targeted use of tyrosine kinase inhibitors for the treatment of CML.

The breakpoints within the ABL1 gene at 9q34 takes place within a large area that spans more than 300 kb at its 5' end, and can occur either upstream of exon 1b, downstream of exon 1a, or, more frequently, between exons 1b and 1a. In contrast, breakpoints within BCR map to three distinct areas known as *breakpoint cluster regions*. In most patients with CML, and in approximately one third of those with Philadelphia chromosome-positive B-cell acute lymphoblastic leukemia (B-ALL), the break occurs within a 5.8-kb area spanning BCR exons e12–e16 (formerly called b1–b5), referred to as the *major breakpoint cluster region* (M-bcr). In two thirds of patients with Philadelphia chromosome-positive B-ALL and in rare cases of CML, the BCR breakpoint localizes to an area of 54.4 kb between exons e2' and e2, termed the “minor breakpoint cluster region” (m-bcr), generating an e1a2 transcript that translates into a 190-kD protein (p190^{BCR-ABL1}). It has been suggested that patients with CML carrying p190^{BCR-ABL1} may have a worse prognosis than those expressing the classical p210^{BCR-ABL1} isoform. A third breakpoint cluster region (*μ-bcr*), downstream of exon 19, has been occasionally identified in patients with CML, giving rise to a 230-kD fusion protein (p230^{BCR-ABL1}), which has been associated with a phenotype similar to that of chronic neutrophilic leukemia.

Anatomy and Autoregulation of the BCR-ABL Protein

The N-terminus of BCR-ABL members consists of the “Cap” region, which is present in two different isoforms generated by alternative splicing of the first exon, termed “1a” and “1b” (Figure 28-1A). ABL 1b contains a C₁₄ myristoyl saturated fatty acid moiety covalently linked to the N-terminus and is expressed at higher levels than type 1a, which is not myristoylated. ABL also contains a tyrosine kinase domain that is preceded by a Src-homology-2

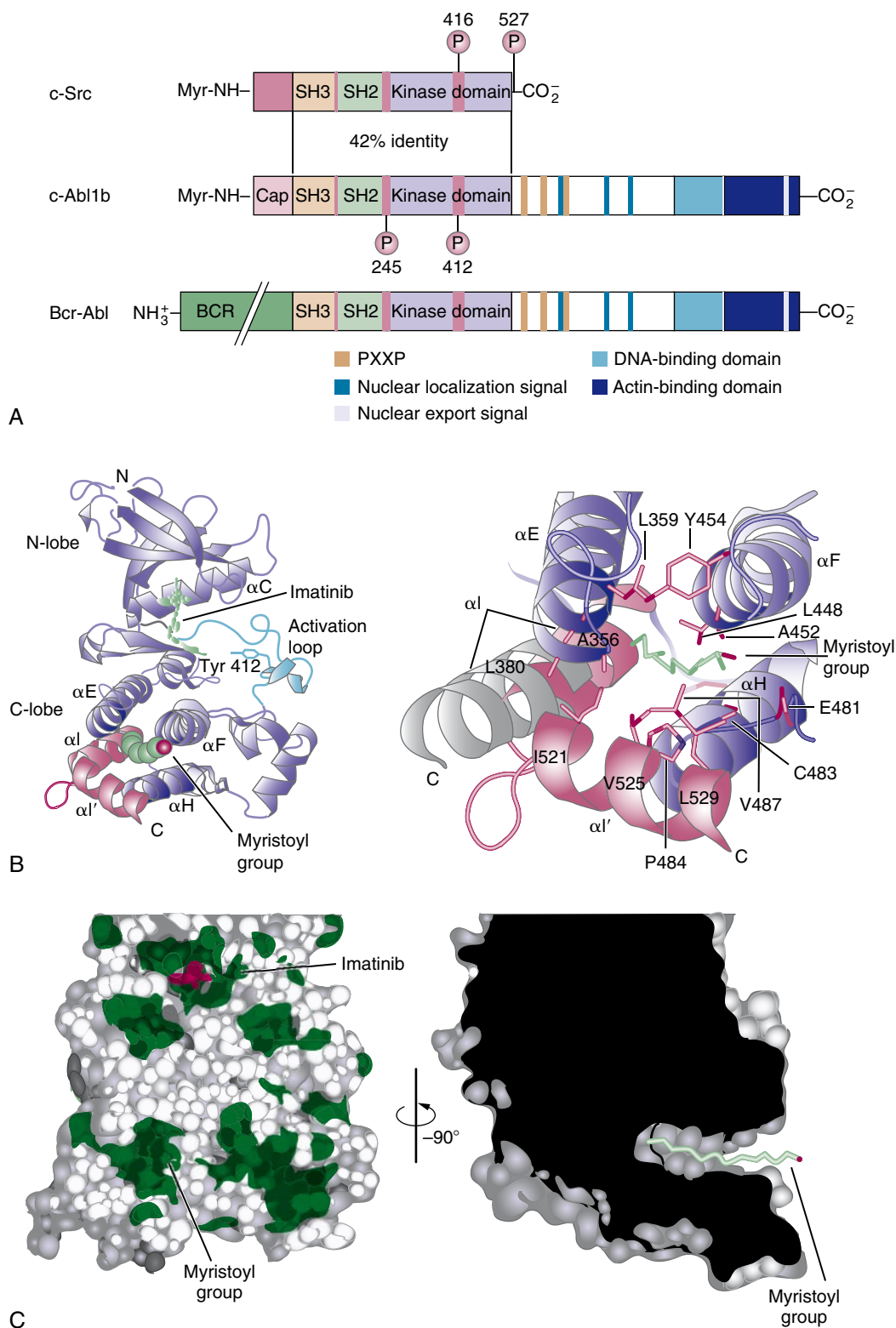


FIGURE 28-1 STRUCTURE OF THE ABL KINASE DOMAIN. **A:** Domain structure of SRC, ABL 1b, and BCR-ABL. Potential tyrosine phosphorylation sites are indicated with red circles and the residue number. **B:** Structure of the ABL kinase domain bound to a myristoylated peptide (*Structure A*). Only the myristoyl group is shown, since the rest of the peptide is disordered. Helices that change conformation upon myristoyl binding (αI and $\alpha I'$) are colored purple. A magnified view of the myristoyl binding site is shown on the right. Superimposed and shown in gray is helix αI from the structure of the isolated kinase domain in the absence of the myristoyl group (PDB code 1M52). **C:** Molecular surface of ABL248–534 (*Structure A*) in the same orientation as in (**B**). Hydrophobic side chains are colored green. A cutaway rendition of the surface is shown on the right. (From Ref. 5, with permission.)

(SH2) and an SH3 domain. These three domains/motifs are highly conserved in terms of sequence and spatial organization. Moreover, ABL has a long C-terminus extension, known as the last exon region, which contains protein–protein interaction sites responsible for the diverse subcellular localizations and functions of the protein. BCR also exhibits a complex architectural modularity at the N-terminus highlighted by the presence of coiled-coil oligomerization domain, a serine/threonine kinase domain, a Dbl/CDC24 guanine-nucleotide exchange factor homology domain and a pleckstrin homology domain, a putative calcium-dependent lipid binding site and a RAC guanosine triphosphatase-activating protein domain.

The Philadelphia (Ph) chromosome causes the replacement of the endogenous autoregulatory domain of ABL with erroneous coding sequences from BCR. In this novel protein, BCR-ABL, the tight temporal and spatial regulation of ABL is lost, resulting in constitutively high levels of tyrosine kinase activity. In BCR-ABL1-positive cells, both SH3 and SH2 domains in ABL function as an auto-inhibitory structure that maintains the kinase domain in an off state (5,6). Crystal structures of ABL have shown that the myristoyl modification at the extreme end of the N-terminal segment of ABL 1b engages with the C-terminal lobe of the ABL catalytic domain (Figures 28-1B and 28-1C). This interaction induces conformational changes that facilitate the docking of the SH2 and SH3 domains onto the kinase domain (7).

Signaling Pathways Downstream of BCR-ABL

The leukemogenic activity of the BCR-ABL fusion tyrosine kinase relies on the activation of numerous downstream effector pathways. The BCR autophosphorylation site Y177 is essential for BCR-ABL1-mediated leukemogenesis. The Y177 residue constitutes a high-affinity docking site for the SH2 domain of growth factor receptor-bound protein 2 (GRB2). GRB2 in turn recruits SOS (a guanine-nucleotide exchanger of RAS) that activates RAS and the scaffold adapter GRB2-associated binding protein 2 (GAB2) through its SH3 domain. Tyrosine phosphorylation of GAB2 in BCR-ABL1-transformed cells requires Y177 of BCR-ABL and the GRB2 SH3 binding site in GAB2. Therefore, BCR-ABL-induced GAB2 phosphorylation is mediated by a GRB2/GAB2 complex. This interaction is required for the full activation of the phosphatidylinositol-3-kinase (PI3K)/AKT and the RAS/ERK pathways as well as for the optimal proliferation and migration of Ba/F3 cells in response to BCR-ABL.

BCR-ABL also phosphorylates the SRC family kinases (SFKs) HCK, LYN, and FGR. Phosphorylation of HCK leads to recruitment of signal transducer and activation of transcription 5 (STAT5), which results in dimerization and translocation of STAT5 molecules to the nucleus where they modulate gene transcription through binding to cognate DNA sequences. Inactivation of STAT5 with siRNA in human samples from patients with CML impairs Ph chromosome-positive myeloid colony formation. Additionally, BCL-X, which is repressed by the transcription factor interferon consensus sequence binding protein (ICSBP), is transcriptionally activated by STAT5 in CML. ICSBP is a tumor suppressor and a negative regulator of granulocyte differentiation.

Forced expression of ICSBP inhibited the BCR-ABL1-induced CML-like disease in vivo.

Although the individual contribution of some downstream elements activated by BCR-ABL may appear negligible when evaluated individually in knock-out systems, there is evidence supporting a cooperative role of some of these factors in the pathogenesis of CML. When BCR-ABL1-positive K562 cells were induced to express dominant negative forms of RAS, PI3K, or STAT5, marked apoptosis was observed in cells coexpressing two of the three dominant negative mutants in any combination. These results suggest that a cooperative interplay among these factors is necessary for the full realization of the leukemogenic potential of BCR-ABL kinase.

Some of the elements involved in BCR-ABL signal transduction provide the rationale for the development of targeted therapies for patients with CML.

Transformation to Blast Phase

Evolution of CML to BP is characterized by the development of a marked degree of resistance to treatment that is difficult to overcome by available therapies. The mechanisms responsible for the transition from the CP of CML to BP remain poorly understood.

Approximately 80% of patients with CML develop additional nonrandom cytogenetic aberrancies in Ph chromosome-positive metaphases. This phenomenon is known as clonal evolution and reflects the genetic instability frequently associated with advanced stages of the disease. The most frequent secondary cytogenetic abnormalities encountered in patients with clonal evolution are trisomy 8 (34%), isochromosome 17 (20%), and duplicate Ph chromosome (38%), which have been linked to c-Myc overexpression, loss of 17p, and BCR-ABL1 overexpression, respectively (12). In addition, 10% to 15% of patients with CML have deletions of the derivative chromosome 9, which may be a reflection of genomic instability leading to more rapid progression to BP than those lacking this abnormality.

Myeloid progenitors from patients with CML in AP or BP have increased β -catenin levels, as compared with levels in controls. Self-renewal of hematopoietic stem cells in mice entails activation of the β -catenin pathway. Of note, CML granulocyte-macrophage progenitors display BCR-ABL1 amplification and activation of the β -catenin pathway, which enhances the self-renewal activity and leukemic potential of these cells (15).

One of the most common gene mutations in CML-BP involves the p53 gene, which is mutated in 25% to 30% of patients with myeloid BP, and exon 2 of the INK4A/ARF locus, which is deleted in 50% of cases of lymphoid BP. Deletion of exon 2 of the INK4A/ARF locus results in loss of p16 and p14/ARF expression, which regulate cell cycle progression and the G₁/S checkpoint by inhibiting the G₁ phase cyclin D–Cdk4/Cdk6 and promoting p53 up-regulation, respectively. Based on the fact that ARF enhances p53 levels by interfering with the activity of MDM2, the principal negative regulator of p53, homozygous deletion at the p16/ARF locus observed in lymphoid BP might represent a functional equivalent of p53 mutation in myeloid BP.

Notably, BCR-ABL enhances SET expression during progression to BP (16). SET is a nucleus/cytoplasm-localized phosphoprotein that potently inhibits the tumor suppressor protein phosphatase 2A (PP2A). In turn, PP2A is a phosphatase that regulates cell proliferation, survival, and differentiation.

ABL Kinase Inhibitor Therapy and Mechanisms of Resistance

The elucidation of the signal transduction network that drives CML culminated in the rational design of imatinib mesylate, an orally bioavailable 2-phenylaminopyrimidine with targeted inhibitory activity against the constitutively active BCR-ABL kinase. Imatinib inhibits the activity of the BCR-ABL kinase with 50% inhibitory concentration (IC_{50}) values ranging between 0.1 and 0.5 μ M (17). After a median follow-up of 60 months, imatinib has been associated with complete hematologic response (CHR) and cytogenetic response (CCyR) rates of 98% and 87%, respectively. However, leukemic residual cells are detectable in most patients with CML receiving imatinib and some of them eventually develop resistance, which is more frequent among those with advanced-phase CML (18). Imatinib resistance has been mainly linked to the development of point mutations within the kinase domain of BCR-ABL1. The most frequent mutations are those that map to the P-loop region of the kinase domain, which serves as a docking site for phosphate moieties of ATP. Imatinib binds to a catalytically inactive conformation of ABL kinase, often referred to as the DFG-out conformation, in which the highly conserved Asp-Phe-Gly (DFG) residues are swung out of their position in the active kinase conformation (19). Imatinib extends deeply into the catalytic domain, and its pyridinyl group locates underneath helix α C in the NH_2 -terminal lobe of ABL kinase. The gatekeeper residue Thr315, located at the periphery of the nucleotide-binding site of ABL, participates in a crucial H-bond interaction between imatinib

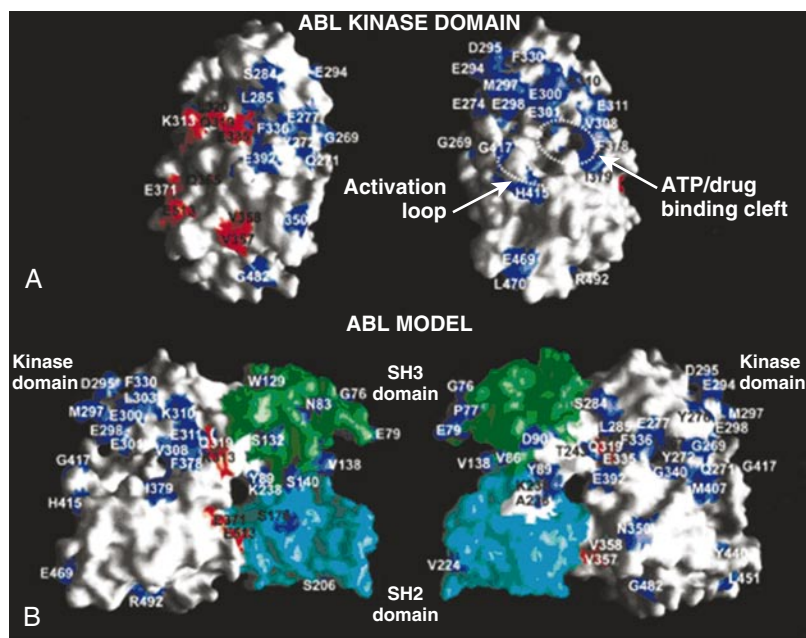
and BCR-ABL (12). Mutation to isoleucine (T315I) impairs the H-bond interaction, which, in addition to the bulk of the isoleucine side-chain, sterically precludes imatinib binding.

The frequency of BCR-ABL1 mutations in imatinib-resistant patients ranges from 40% to 90%, depending on the methodology of detection, CML phase, and the definition of resistance (20,21). More than 50 distinct point mutations encoding for single-amino-acid substitutions in the kinase domain of BCR-ABL1 have been detected (Figure 28-2) (22). ABL kinase domain mutations result in distortion of the ABL kinase/imatinib interface and preclude the adoption of the inactive conformation of the kinase, thus impairing imatinib binding (Figure 28-3). However, most identified mutations are rare, with mutants affecting residues Gly250, Tyr253, Glu255, Thr315, Met351, and Phe359 accounting for 60% to 70% of all mutations. Finally, the resistance conferred by most ABL kinase mutants, except for T315I, can be overcome by the second-generation ABL kinase inhibitors nilotinib (25), and dasatinib (Figures 28-4 and 28-5; 26). This justifies the continued development of potent BCR-ABL inhibitors with distinct mutagenicity profiles, and the use of these agents in combination to minimize the development of resistance.

The Challenging T315I Mutation

BCR-ABL1 T315I mediates complete resistance to imatinib in a subset of patients with CML harboring mutations within the kinase domain of ABL and constitutes a formidable challenge (27). Topographically, the T315I mutation affects a highly conserved “gatekeeper” threonine residue near the ABL catalytic domain, which controls the access to a hydrophobic region of the enzymatic active site that is not contacted by ATP, causing steric clash owing to the physical constraints imposed by the voluminous isoleucine residue. Several strategies have been pursued aiming to override T315I-mediated imatinib resistance that include modeling novel

FIGURE 28-2 MAPPING OF MUTATIONS TO STRUCTURAL MODELS OF ABL. **A:** Opposite views of the ABL kinase domain surface, encompassing amino acid residues 76–517 (57–498 by type Ia numbering, PDB 1IEP). Highlighted in blue or red are the positions of kinase domain mutations isolated in this study. Mutated residues that mediate contacts with either SH3 or SH2 are colored red. **B:** Opposite views of the predicted surface of a composite ABL kinase (PDB 1IEP) (From Ref. 14, with permission.), SH3/SH2 (PDB 2ABL; Ref. 15), and CD-linker model. The two perspectives of the composite ABL SH3/SH2/kinase model are colored as in (A). The SH3 domain is shown in green, the SH2 domain in cyan, and the CD linker in white. (From Ref. 14, with permission.) (From Ref. xx, with permission.)



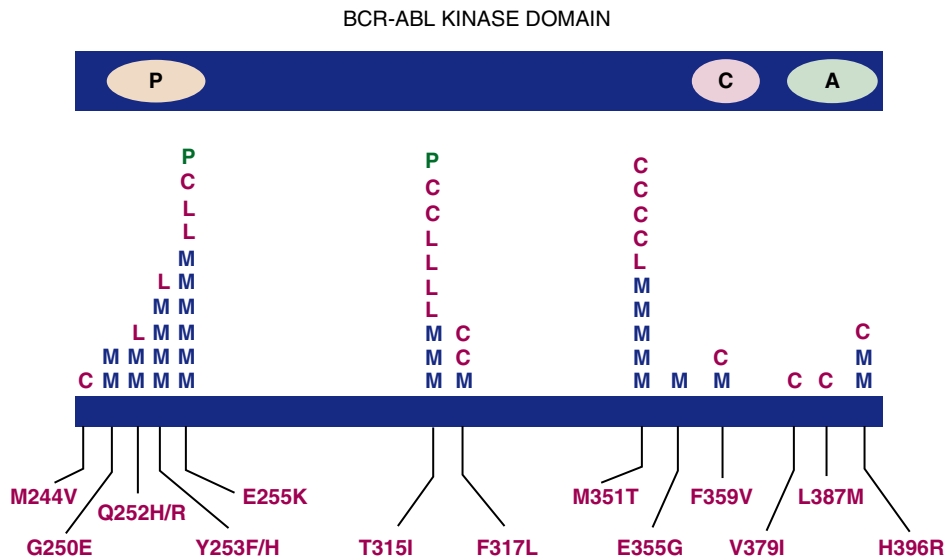


FIGURE 28-3 CLINICALLY RELEVANT IMATINIB-RESISTANT BCR-ABL KINASE DOMAIN MUTATIONS. **Top:** Schema of the BCR-ABL kinase domain depicting the P loop (P), catalytic domain (C), and activation loop (A). **Bottom:** Each letter represents a patient in whom the corresponding mutation was detected. Relapsed chronic-phase patients are represented by the letter C (for exception, see below). Relapsed myeloid blast crisis patients are indicated by the letter M. Patients with relapsed lymphoid blast crisis are represented by the letter L. P indicates mutations detected before imatinib treatment in patients with myeloid blast crisis who exhibited primary resistance to treatment. (From Ref. xx, with permission.)

small-molecule inhibitors that accommodate the structural constraints imposed by this mutant and the development of compounds that target binding sites outside the ATP-binding domain of BCR-ABL. Among the first type of agents is the Aurora kinase inhibitor MK-0457 (formerly VX-680), which inhibits BCR-ABL T315I with an IC_{50} of 5 nM.

Acute Myeloid Leukemia

AML is a heterogeneous disease with regard to acquired genetic alterations, including cytogenetic aberrancies, gene mutations, and changes in gene expression. Most cases of AML are sporadic and occur as a consequence of acquired somatic mutation in hematopoietic stem cells (Figure 28-6). However, there is significant variation in clinical outcome across different cytogenetic aberrations. Acquired structural chromosome aberrancies are detected at diagnosis in the bone marrow of 50% to 60% of patients with AML (Table 28-1) (29). Three distinct risk groups, favorable, intermediate, and adverse, can be established on the basis of karyotypic findings. All large cytogenetic studies of AML concur that patients with $t(15;17)(q22;q12-21)$ have an excellent prognosis and those with $t(8;21)(q22;q22)$ or $inv(16)(p13q22)/t(16;16)(p13;q22)$ have a relatively favorable prognosis. By contrast, patients with $inv(3)(q21q26)/t(3;3)(q21;q26)$, -7 or a complex karyotype, (i.e., at least three chromosome aberrations), have a poor clinical outcome. The presence of the poor-risk cytogenetic abnormalities -5 , $del(7q)$, $-17/17p$, -18 , or -20 , are frequently associated with complex karyotypes.

AML Pathogenesis

In AML, the enormous complexity and diversity of genetic and molecular genotypes exceed the number of recognizable clinical and morphologic phenotypes. A working formulation that rationally categorizes the wealth of available genetic and emerging

molecular information is therefore necessary. An accepted notion of AML pathogenesis involves the cooperation of two types of genetic abnormalities/mutations. The most frequently mutated genes in AML encode transcription factors, thus emphasizing the critical role of these “master” regulators in the control of hematopoietic cell development. A direct consequence of these mutations is the impairment of the processes of maturation and differentiation of cells of the myeloid lineage. A second type of mutation are those that confer a proliferative and survival advantage to cells (activating mutations) (30). An example of the former includes the core binding factor (CBF) translocations and *RAR α* and *MLL* gene rearrangements. Examples of the latter include mutations in receptor tyrosine kinases such as KIT or the *fms*-related tyrosine kinase 3 (FLT3) genes. Some of the mutations conferring proliferative and survival advantage correlate with karyotypic aberrations observed in patients with AML at diagnosis (Table 28-2).

Mutations Interfering with Transcription in AML

Two distinct groups of transcriptionally active proteins play a major role in the fate of hematopoietic progenitors. The first group consists of master regulatory transcription factors, which, like AML1, are implicated in the development of all the hematopoietic lineages. A second category of transcription factors intervene more specifically in the development and each hematopoietic lineage. Examples of this type of transcription factors are GATA-1, which skews the development of hematopoietic progenitors toward the erythroid lineage, or C/EBP α , which promotes granulocytic differentiation.

Core Binding Factor Rearrangements

Mutations involving CBF rearrangements are detectable in approximately 15% of cases of AML and frequently confer a favorable prognosis. CBF is a heterodimeric transcription factor that consists of a DNA binding α -subunit, encoded by one of three members of

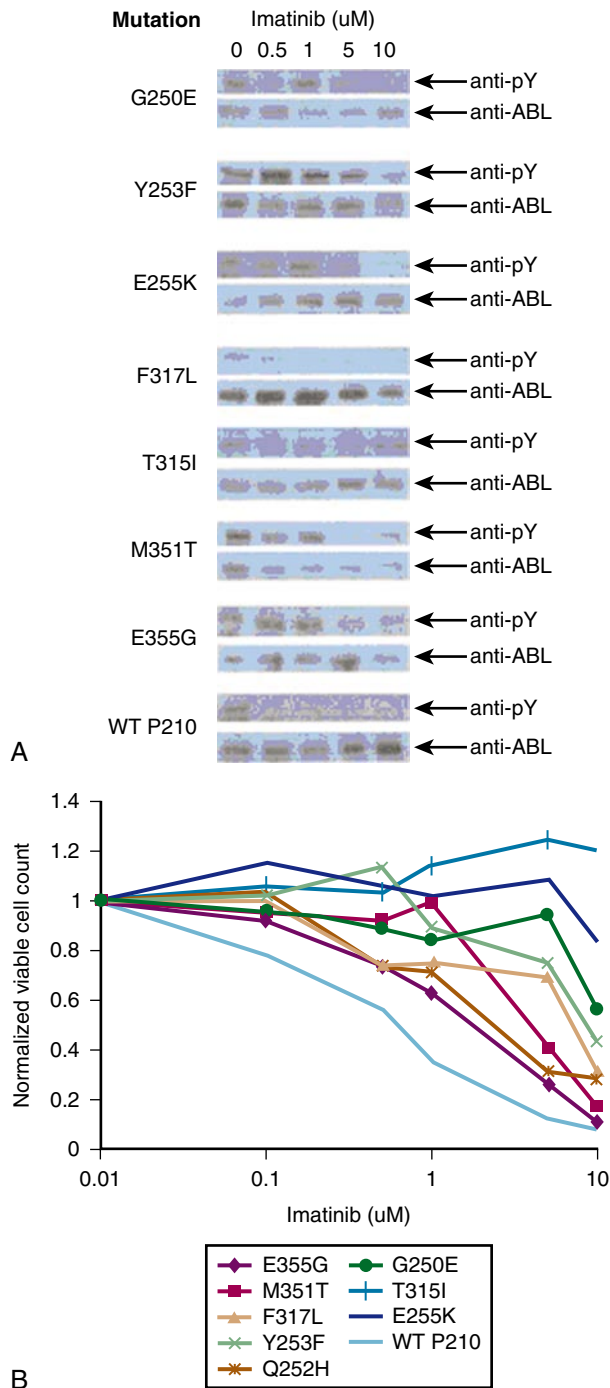


FIGURE 28-4 BCR-ABL KINASE DOMAIN MUTANTS EXHIBIT VARYING DEGREES OF RESISTANCE TO IMATINIB. **A:** Western blot analysis using an antiphosphotyrosine antibody (4G10) or anti-ABL antibody of lysates prepared from interleukin-3 (IL-3)-independent Ba/F3 populations retrovirally transfected with the BCR-ABL1 isoforms indicated. Cells were exposed to imatinib for 2 hours at the indicated concentrations. **B:** Ba/F3 populations stably transfected with mutant BCR-ABL1 isoforms were cultured in the presence of varying concentrations of imatinib for 48 hours. Cell counts were normalized to the number of viable cells from control cultures grown in the absence of imatinib. (From Ref. xx, with permission.)

the RUNX family (*RUNX1* or *AML1*, *RUNX2*, and *RUNX3*), and a β -subunit encoded by the *CBF β* gene that increases DNA-binding affinity to the complex. Rearrangements of *AML1* and *CBF β* with other genes result in chimeric proteins that disrupt

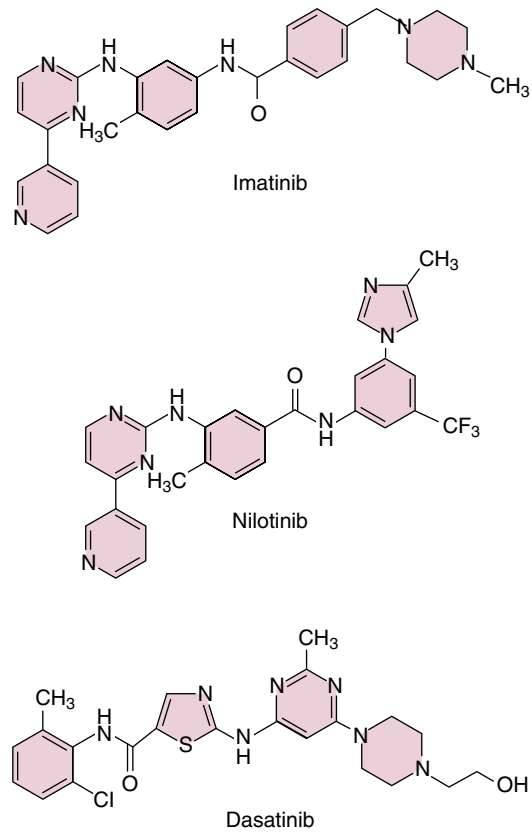


FIGURE 28-5 Molecular structures of imatinib and second-generation tyrosine kinase inhibitors nilotinib and dasatinib.

the CBF complex and represses transcription activation. A variety of chromosomal translocations involving either *AML1* or *CBF β* have been identified in AML. CBF AML include those expressing *inv(16)/t(16;16)*, which gives rise to the fusion of *CBF β* with the smooth muscled myosin heavy chain gene (*MYH11* or *SMMHC*), and *t(8;21)*, which is associated with the fusion transcript composed of the *AML1* and the eight-twenty-one (*ETO*) genes. The *AML1* gene has been found implicated in other fusion transcripts detected in AML, such as those involving the ecotropic viral integration 1 (*EV11*), or translocation ets leukemia (*TEL*) genes in AML carrying the *t(3;21)* and *t(12;21)* translocations, respectively. In AML expressing the CBF translocation *AML1-ETO*, this fusion oncogene acts as a dominant negative inhibitor of *AML1* (Figure 28-7), as evidenced in transcriptional activation assays. In summary, genetic rearrangements of CBF are frequent in patients with AML and usually occur through balanced reciprocal translocations, accounting for approximately 25% of cases of all cases of AML.

PML-RARa Rearrangements

Translocations involving the *retinoic acid receptor* (*RAR*) locus on chromosome 17, such as *t(15;17)(q22;q11)*, are linked to the phenotype of acute promyelocytic leukemia (APL), which comprises 10% to 15% of all cases of adult AML (33). The *t(15;17)(q22;q11)* gives rise to the *PML-RARa* transcript that encodes a fusion protein containing most of the functional domains of *RARa* (including the *RAR* binding domain and the

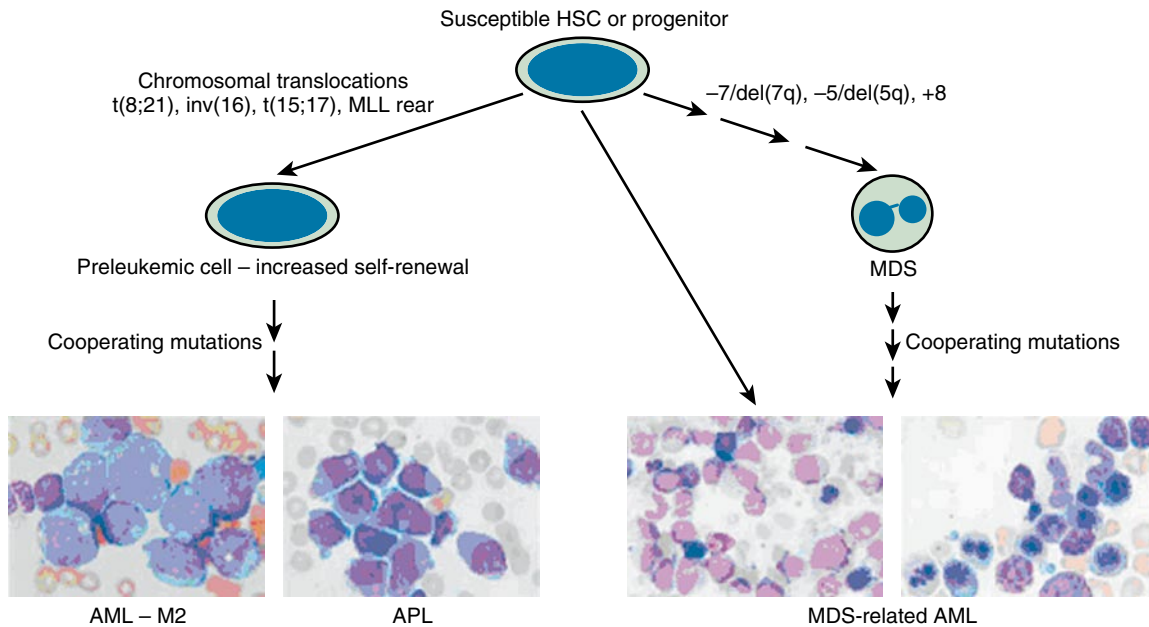


FIGURE 28-6 PATHOGENESIS OF ACUTE MYELOID LEUKEMIA (AML), MYELODYSPLASTIC SYNDROME (MDS), AND MDS-RELATED AML. During ontogeny, hematopoietic stem cells (HSCs) may acquire certain chromosomal translocations or rearrangements that result in the production of fusion oncoproteins that typically lead to the enhanced self-renewal capacity of HSC and their committed progenitors. These mutated HSCs serve as a silent preleukemic population that can acquire other cooperating mutations leading to the formation of an overt leukemic clone. Two examples of this subtype of leukemia are AML harboring the t(8;21) translocation (M2 of the French-American-British classification) and t(15;17)-containing acute promyelocytic leukemia (APL). By contrast, MDS is likely to arise through the acquisition of multiple mutations. Once established, the myelodysplastic stem cell can continue to undergo additional genetic and epigenetic changes, which in a substantial percentage of cases will lead to the development of overt AML. (From Ref. 22, with permission.)

Table 28-1 Frequent Chromosomal Aberrations in AML

	Genes	Morphological Association	Incidence*
Translocations/Inversions			
t(8;21)(q22;q22)	<i>RUNX1;RUNX1T1</i>	M2 with Auer rods	6%
inv(16)(p13q22) or t(16;16)(p13;q22)	<i>CBFB;MYH11</i>	M4Eo	7%
t(15;17)(q22;q11-21)	<i>PML;RARA</i>	M3/M3v	7%
t(9;11)(p22;q23)	<i>MLL;AF9</i>	M5	2%
t(6;11)(q27;q23)	<i>MLL;AF6</i>	M4 and M5	~1%
inv(3)(q21q26) or t(3;3)(q21;q26)	<i>EVI;RPN1</i>	M1, M4, M6, M7?	~1%
t(6;9)(p23;q34)	<i>DEK;NUP214</i>	M2, M4	~1%
Chromosomal Imbalances			
+8	..	M2, M4 and M5	9%
-7/7q-	..	No FAB preference	7%
-5/5q-	..	No FAB preference	7%
-17/17p-	TP53	No FAB preference	5%
-20/20q-	..	No FAB preference	3%
9q-	..	No FAB preference	3%

(Continued)

Table 28-1 Frequent Chromosomal Aberrations in AML—(Continued)

	Genes	Morphological Association	Incidence*
Chromosomal Imbalances			
+22	..	M4, M4Eo	3%
+21	..	No FAB preference	2%
+13	..	Mo, M1	2%
+11	MLL1 [†]	M1, M2	2%
Complex karyotype [‡]			10%
Normal karyotype			44%

AML, acute myeloid leukemia.
* Determined among 1,311 patients with *de novo* AML enrolled onto Study 8461 of the Cancer and Leukemia Group B.
[†] Partial tandem duplication of the *MLL1*.
[‡] Three or more chromosomal aberrations in the absence of t(8;21), inv(16)/t(16;16), t(15;17), or t(9;11).
Source: Adapted from Dohner K, Mrozek K, Dohner H, Bloomfield CD. *Leukemia Diagnosis and Classifications*. Amsterdam: Elsevier Inc., (in press).

DNA binding domain) and the majority of the *PML* gene. The breakpoints within the *PML* gene cluster locate to three different regions referred to as bcr1, bcr12, and bcr13.

In addition to t(15;17)(q22;q11), there are at least two other variant translocations involving *RARα* associated with the APL phenotype. These include t(11;17)(q23;q21) and t(5;17)(q35;q21), which lead to the fusion of the *RARα* gene to the promyelocytic leukemia zinc finger (*PLZF*) and nucleophosmin (*NPM*) genes, respectively. Other genes, such as *NuMA* and *STAT5*, have been found as much rarer *PML* translocation partners.

All-*trans* retinoic acid (ATRA) is the mainstay of the treatment for patients with APL, inducing leukemic cell differen-

tiation and remission in patients with t(15;17)/*PML-RARα* or t(5;17)/*NPM-RARα* (35). Similarly, arsenic trioxide (As₂O₃) has been demonstrated to be effective in the treatment of *de novo* and ATRA-resistant t(15;17)/*PML-RARα* APL (36). Interestingly, patients with APL harboring t(15;17)(q22;q11)/*PLZF-RARα* fail to respond to ATRA. Paradoxically, both *PML-RARα* and *PLZF-RARα* contain identical *RAR* sequences and inhibit ATRA-induced gene transcription and cell differentiation. Both fusion proteins recruit the nuclear corepressor (N-CoR)–histone deacetylase complex through the *RARα* CoR box. Although ATRA can induce dissociation of N-CoR from *PML-RARα*, it does not affect its association with the *PLZF-RARα* fusion

Table 28-2 Association of Karyotypic Aberrations with Molecular Findings in AML

	Molecular Genetic Defect	Prevalence	Study
t(8;21)	KIT exon 8 mutation	2%	21
	KIT codon 816 mutation	11%	21,25
	FLT3 ITD or D835 mutation	6–11%	21,25
inv(16)/t(16;16)*	KIT exon 8 mutation	24–26%	21,23
	KIT codon 816 mutation	7–8%	21,23
	FLT3 ITD or D835 mutation	8%	21,23
	NRAS mutation	18–26%	23,28
	KRAS mutation	9–17%	23,28
Normal karyotype	FLT3 ITD	28–34%	73–76
	FLT3 TKD mutation	11–14%	74,75
	NPM1 mutation	48–64%	79–83
	CEBPA mutation	15–18%	87,99
	MLL1 PTD	8–11%	89,90
	NRAS mutation	14%	28
	KRAS mutation	4%	28 BAALC
gene overexpression	..	..	98
t(6;9)	FLT3 ITD	90% [†]	75
+11	MLL PTD	91% [†]	94
+21	RUNX1 mutation	38% [†]	92
del(9q)	CEBPA mutation	41%	93

AML, acute myeloid leukemia.
* ≤70% of inv(16) leukemias have mutations in receptor tyrosine kinase or *RAS* genes.
[†] Prevalence based on a limited number of cases.
Source: Adapted from Estey, et al. *Lancet* 2006;368:1894–1907, with permission.

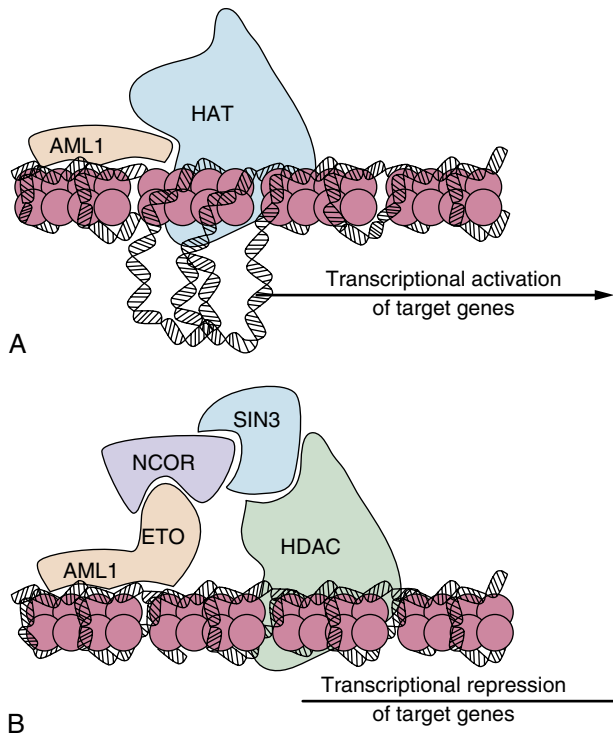


FIGURE 28-7 AML-ETO ACTS AS A DOMINANT NEGATIVE INHIBITOR OF AML1. **A:** Regulation in normal hematopoietic progenitors. AML1 recruits co-activators upon binding to its target genes. The histone acetyl transferase activity of the co-activators causes an open chromatin structure, facilitating the transcription of AML1 target genes. **B:** Regulation AML1-ETO-expressing leukemic cells. ETO recruits corepressors and consequently histone deacetylases to AML1 DNA-binding sites. As a result, the DNA structure is closed, thus causing transcriptional repression of AML1 target genes. HAT, histone acetyl transferases; HDAC, histone deacetylases. (From Ref. 20, with permission.)

protein, thus resulting in persistent block in gene expression and hematopoietic differentiation (Figure 28-8). This insensitivity is mediated by the N-terminal PLZF moiety of the chimera.

Mixed-Lineage Leukemia Gene Rearrangements

Mixed-lineage leukemia (MLL), also known as *ALL-1*, *HRX*, or *HTRX1*, is the human homologue of *Drosophila TRX* and constitutes a maintenance factor for the homeobox (HOX) group of proteins, which are central to cell fate during development and hematopoiesis. The N-terminus of *MLL*, which contains the AT-hook DNA-binding motif and a region homologous to DNA methyltransferase, is always retained in the fusion protein whereas the C-terminus, which contains the activation and SET domains, is always replaced by the fusion partner. Approximately 4% of patients with de novo AML have balanced translocations or insertions involving the *MLL* gene. *MLL* gene fusions are highly associated to previous therapy with topoisomerase-II inhibitors such as etoposide, and to the monocytic FAB-subtype M5a.

In AML, more than 65 different chromosomal regions have been thus far identified as *MLL* fusion partners. All *MLL* rearrangements contain invariably a truncated *MLL* gene product expressed from its own promoter. In addition, some patients with AML have rearrangements of *MLL* in which *MLL* is not fused with a partner gene but rather is elongated by means of an in-frame partial tandem duplication (*MLL*-PTD) of exons 11–5 or 12–5. *MLL*-PTD occurs chiefly in normal karyotype AML or in trisomy 11 and can be identified in 5% to 10% of all AML cases. Approximately 4% to 7% of patients with AML with normal cytogenetics carry *MLL*-PTD, which confers an especially poor prognosis.

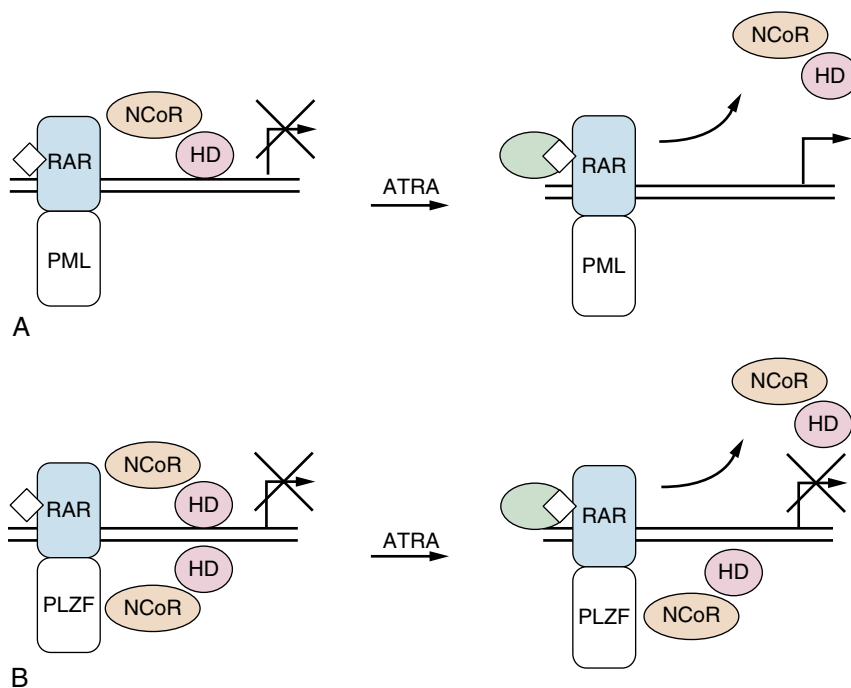


FIGURE 28-8 PML-RARA INTERACTS WITH NUCLEAR CO-REPRESSOR (NCoR) HISTONE DEACETYLASE (HD) COMPLEX. **A:** All-*trans* retinoic acid (ATRA) causes dissociation of the corepressor complex and release of transactivation. **B:** In cells expressing the PLZF-RARA fusion oncogene, the PLZF moiety recruits the NCoR/HD complex, rendering PLZF-RARA-mediated repression unresponsive to ATRA. (From Ref. xx, with permission.)

Rearrangements Involving *HOX* Genes

HOX genes are frequently overexpressed in leukemia (37). *HOX* genes are expressed in early hematopoietic progenitors but are undetectable in terminally differentiated cells. Gene expression profiling analysis showed that the *HOXA4*, *HOXA9*, *HOXA10*, *PBX3*, and *MEIS1* homeobox genes are coexpressed across diverse cytogenetic groups, suggesting a coregulated pathway with pathologic relevance in a subgroup of AML (38). *HOX* genes are mainly disrupted via chromosomal translocation. For instance, *HOXA9* and *HOXD13* are deregulated through the t(7;11) and t(2;11) translocations, respectively, giving rise to fusion proteins between the *HOX* protein and the nucleoporin 98-kD (NUP98) nuclear protein (39). The NUP98 moiety of *HOXA9*-NUP98 has transactivating capacity and interacts with the transcriptional coactivator c/EBP binding protein (CBP)/p300. Overexpression of *HOXA6*, *HOXA7*, *HOXA9*, and the *HOX* cofactor myeloid ecotropic viral integration site 1 (*MEIS1*) has also been correlated with chromosome 11q23 abnormalities involving the MLL protein, which directly regulates the expression of *HOX* genes.

Mutations in the *C/EBPα* Gene

The *C/EBPα* gene, which encodes the CCAAT/enhancer-binding-protein- α , is a member of the family of leucine-zipper (bZIP)-transcription factors that couples lineage commitment to terminal differentiation and cell cycle arrest in the process of myeloid differentiation (40). *C/EBPα* initiates growth arrest through induction of *p21* and by disrupting the E2F transcriptional complexes during the G₁ phase of the cell cycle. Mutations in the *C/EBPα* gene occur in 15% to 19% of patients with AML and normal cytogenetics. In the absence of specific *C/EBPα* mutations, decreased expression may serve as an alternative mechanism that disrupts *C/EBPα* gene function. Importantly, *C/EBPα* mutations have been shown to be associated with a favorable prognosis in patients in the cytogenetic intermediate-risk category and may improve risk stratification in patients with normal cytogenetics.

Mutations in the *PU.1* Gene

The transcription factor *PU.1* encoded by the *Sfpi1* (spleen focus-forming virus proviral integration) gene is indispensable for myelomonocytic differentiation during normal hematopoiesis and for regulating the commitment of multipotent hematopoietic progenitors. Aberrant levels of expression of *PU.1* are leukemogenic. Transcriptional control of *Sfpi1* gene expression is regulated by a distal upstream regulatory element (URE) that is highly conserved. Knock out of URE reduces expression of *PU.1* by 80% in the bone marrow and leads to the development of AML in mice (42). This suggests that reduced *PU.1* levels are sufficient to support the survival of myeloid progenitors but not to sustain their differentiation, possibly due to deregulation of the expression of some cytokine receptors. Repressed *PU.1* transcription has been reported in AML expressing *PML-RARα* or internal tandem duplication *FLT3* (*FLT3-ITD*) mutations, and *AML1-ETO* functionally inactivates *PU.1* by displacing its coactivator JUN.

Mutations Altering Signal Transduction

Mutations of the Fms-Related Tyrosine Kinase 3 (*FLT3*) Gene

The *FLT3* gene locates to chromosome band 13q12 and encodes a membrane-bound structurally related to other class III tyrosine kinase receptors such as KIT, FMS, and PDGFR. In the bone marrow, *FLT3* expression is restricted to CD34⁺ cells and a subset of dendritic precursors, where it regulates proliferation, differentiation, and apoptosis of hematopoietic cell progenitors. A series of models have investigated the role of *FLT3* signaling in leukemogenesis. Internal tandem duplication (ITD) of the *FLT3* gene, occurring within the *FLT3* juxtamembrane domain (exons 14 and 15), are among the most prevalent mutations detected in patients with AML and normal karyotype, being detected in about 30% of cases (44). Moreover, 7% of patients with AML harbor missense point mutations affecting the activation loop of the tyrosine kinase domain of *FLT3* coded by exon 20 (Figure 28-9). A variety of kinase domain mutations have been reported. *FLT3-ITD* and *FLT3* kinase domain mutations give rise to a constitutively active *FLT3* protein that promotes ligand-independent proliferation and survival of leukemic cells. Oncogenic activation of *FLT3-ITD* mutations activates aberrant signaling including STAT5 and repression of myeloid transcription factors such as *PU.1* and *C/EBPα*. In addition to STAT5, *FLT3* signals through the PI3K/AKT and the MAPK pathways. Small-molecule tyrosine kinase inhibitors directed against the constitutively activated *FLT3* protein, such as PKC412, lestaurtinib, sorafenib, and tandutinib, have shown encouraging results in early clinical trials, although complete responses have been rarely achieved.

Mutations of the *KIT* Gene

The *KIT* gene, located at chromosome band 4q11–12, encodes a 145-kD transmembrane receptor tyrosine kinase of the type III subgroup. Upon binding of the ligand stem cell factor (SCF) to the extracellular immunoglobulin-like domains, KIT undergoes homodimerization and autophosphorylation at the Y568 and Y570 tyrosine residues of the juxtamembrane domain, which activates downstream signaling pathways involved in proliferation, differentiation, and survival. Gain-of-function point mutations in the kinase domain of KIT result in ligand-independent constitutive activation of KIT signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis. Activation of the KIT tyrosine kinase by somatic mutation has been documented in a variety of human malignancies, including core binding factor AML, systemic mastocytosis, and gastrointestinal stromal tumors. In AML, most *KIT* mutations cluster within exon 17, which encodes the KIT activation loop (A-loop) in the kinase domain, and in exon 8, which encodes a highly conserved region in the extracellular portion of the KIT receptor, which is believed to play a role in receptor dimerization (46, 47). Interestingly, *KIT* mutations at exon 17 in patients with inv(16) occur exclusively at codon D816, whereas in patients with t(8;21), they map

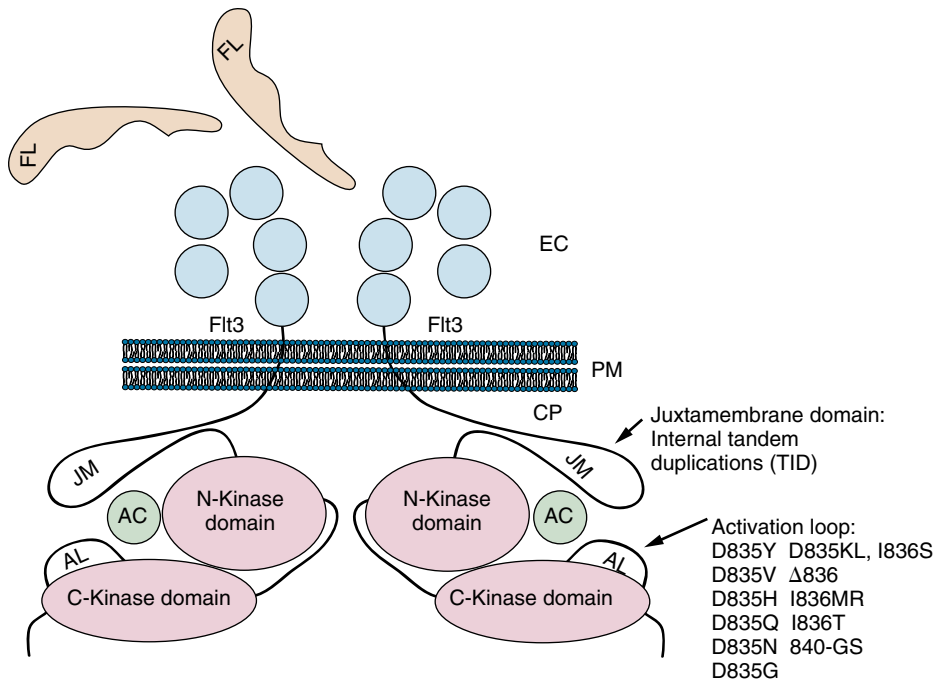


FIGURE 28-9 FLT3-ACTIVATING MUTATIONS. The schema represents the FLT3 protein kinase in the autoinhibitory state, prior to binding of the ligand to the extracellular (EC) domain. In this state, the active center (AC) of the kinase is obstructed by sequences of the juxtamembrane domain (JM) and of the activation loop (AL). Ligand-induced activation of the receptor results in the phosphorylation of critical tyrosine residues in the JM and AL regions of the receptor and the release of the AC. The activating mutations occur as internal tandem duplications (ITDs) in the JM domain as well as point mutations in the AL and are thought to disrupt their function. A series of reported point mutations are indicated. CP, cytoplasm; FL, FLT3-ligand; PM, plasma membrane. (From Ref. 20, with permission.)

primarily to codon D816 or N822. Indeed, variable responses to imatinib administered both as single agent or in combination with other chemotherapeutics have been reported in core binding factor AML with *KIT* mutations at exon 8, whereas patients with *KIT* D816 mutations did not show any response. In vitro studies have shown that *KIT* D816 mutations can be effectively targeted with tyrosine kinase inhibitors such as PKC412, or dasatinib.

Mutations of the RAS Gene

Abnormalities in RAS genes have been implicated in the pathogenesis of AML. Mutations in all RAS gene homologues, *NRAS*, *KRAS*, and *HRAS*, take place at codons 12, 13, and 61. All RAS proteins possess a consensus guanosine triphosphate (GTP) binding motif that critical for intracellular signaling. The RAS proteins oscillate between a GTP- and a guanosine diphosphate (GDP)-bound state. GDP-bound RAS is incapable of activating signal transduction pathways (48). *NRAS* mutations are the most frequent RAS mutations in AML, being detected in 10% to 30% of patients.

RAS may represent a potential therapeutic target in AML. Farnesyl transferase inhibitors (FTIs) constitute a class of drugs that targets the farnesylation of RAS proteins (required for the attachment of this protein to the plasma membrane) (49). This post-translational modification is critical for RAS-mediated cell transformation. However, the nonpeptidomimetic FTI tipifarnib used in combination with standard idarubicin and cytarabine induction chemotherapy has not improved the results compared with historical controls in a recently reported study.

Other Genetic Events Implicated in the Pathogenesis of AML

Mutations of the Nucleophosmin Gene

The nucleophosmin member 1 (*NPM1*) gene located at chromosome band 5q35, encodes a nucleus-cytoplasm shuttling protein implicated in preventing nucleolar protein aggregation, regulation of ribosomal protein assembly, initiation of centrosome duplication, and regulation of the oncosuppressors *p53* and *p19ARF* and their partners (i.e., *HDM2/MDM2*). Mutations of *NPM1* result in the shift of the *NPM1* protein into the cytoplasm and constitute the most frequent genetic aberration found in AML with normal karyotype. Given that *NPM1* is thought to have a tumor-suppressor function, alterations in its subcellular localization from the nucleus to the cytoplasm may be crucial for malignant transformation. *NPM1* mutations are found in approximately 35% of adult patients with primary AML (excluding PML) and have been linked to a higher complete remission rate after induction chemotherapy. Most of the cases (60%–80%) carrying a mutant *NPM1* gene correspond to patients with AML and normal karyotype (50). *NPM1* mutations are typically heterozygous and are restricted to exon 12. Since immunohistochemical detection of cytoplasmic *NPM1* is predictive of *NPM1* mutations in AML, and these are associated with a better prognosis, the detection of *NPM1* mutations may represent a valuable tool both for outcome prediction as well as for monitoring of minimal residual disease.

Overexpression of the *BAALC* and *ERG* Genes

High expression of the brain and acute leukemia cytoplasmic (*BAALC*) gene can be found in some patients with AML, which

has been shown to portend an adverse prognosis in patients younger than 60 years of age. In one study including 307 patients younger than 60 years with AML and normal karyotype, *BAALC* overexpression predicted for failure to achieve complete response, primary resistant disease, and remarkably shorter overall survival. Another study suggested that high *BAALC* expression may represent a prognostic marker particularly useful in AML with normal karyotype lacking *FLT3*-ITD and *C/EBP α* mutations.

The v-ets erythroblastosis virus E26 oncogene homologue (avian) (*ERG*) gene maps to chromosome band 21q22 and is involved in regulating and promoting cell differentiation, proliferation, and tissue invasion. Patients with AML and normal karyotype expressing the highest *ERG* levels have a worse cumulative incidence of relapse than those with low expression ($p < 0.001$), particularly in those with low *BAALC* expression.

Conclusion

Significant advances have been made regarding the understanding of the mechanisms involved in the pathogenesis of myeloid

leukemias. The development of targeted therapies against myeloid malignancies is epitomized by the remarkable success of the small-molecule kinase inhibitor imatinib mesylate in patients with CML. However, *BCR-ABL1* transcripts are detectable in most patients receiving imatinib, indicating that single-agent therapy with this tyrosine kinase inhibitor may not cure CML. It is likely that the complete eradication of leukemic cells in CML will be accomplished by using combination approaches.

In AML, the discovery that mutation-induced deregulation of several tyrosine kinase receptors (e.g., *KIT*, *FLT3*) provides a survival and proliferative advantage to AML cells has opened the avenue of targeted therapies in this disease. The efficacy of ATRA and arsenic trioxide in PML not only conveys the hope that therapy for other types of AML may diverge from the current practice of administration of cytotoxic agents, but also underscores the therapeutic potential of targeting transcription factors for the treatment of AML. Translocation-generated oncogenic fusion proteins involving transcription factors provide novel targets for therapy. However, designing therapeutic modalities aimed at modulating transcription factors remains challenging.

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Molecular Biology of Childhood Neoplasms

Cancer is the most common cause of disease-related death in children beyond the newborn period. Although childhood cancers, as a group, account for only a small proportion of all human cancer (Figure 29-1), their unique biologic features, cell of origin, and response to therapy make them intriguing models with which to study and understand the process of human carcinogenesis.

Most childhood cancers occur sporadically and their etiology remains unclear. Obvious environmental influences to cancer initiation are not generally apparent. Cancer predisposition syndromes manifesting in childhood in which nonmalignant phenotypic features are not observed include hereditary retinoblastoma (RB), Li-Fraumeni syndrome, and familial polyposis, while others such as von Hippel Lindau disease are associated with the coincident presentation of both benign and malignant neoplasms. Nonrandom molecular and cytogenetic alterations are frequently observed in most childhood cancers. These markers not only provide unique diagnostic identifiers, but also frequently provide some prognostic value with respect to disease outcome and anticipated response to therapy. Importantly, many of these genetic “markers” also recapitulate normal developmental processes, and as such, offer a window into the biologic mechanisms of carcinogenesis and normal embryologic growth and development (Table 29-1). In this chapter, we address the diversity of molecular mechanisms in several prototypical childhood cancers and cancer predisposition syndromes.

Retinoblastoma

Clinical Description and Pathology

Retinoblastoma (RB) is a rare childhood tumor thought to arise in the embryonic retinal epithelium. Although the incidence of RB is only approximately 1 in 20,000 births, or some 200 new cases per year in North America (2), this tumor has been a target of intense research interest. RB is the prototype cancer caused by mutations of a tumor suppressor gene. Tumors are often bilateral and multifocal. In approximately 40% of RB cases, the disease is inherited as an autosomal dominant trait, with a penetrance approaching 100% (3). The remaining 60% of retinoblastoma cases are sporadic (nonheritable). Fifteen percent of unilateral

RB is heritable but by chance develops in only one eye. Survivors of heritable retinoblastoma have a 100-fold increased risk of developing mesenchymal tumors such as osteogenic sarcoma, fibrosarcoma, and melanoma later in life. RB is characterized by the rapid growth of undifferentiated neuroblastic precursors derived from various layers of retinal ganglion cells. The cells are small and round with a high nuclear: cytoplasmic ratio, exhibiting numerous mitoses that reflect their rapid proliferative rate. RB cells appear undifferentiated, with evidence of ganglionic differentiation, including the presence of Flexner-Wintersteiner rosettes. Electron microscopy frequently reveals structural features unique to retinal cells.

Tumors that are limited to the globe are staged according to the schema of Reese and Ellsworth, which is based on the number and size of the tumors (4). This classification predicts the likelihood of obtaining local tumor control and preservation of vision. Each eye is staged individually. RB can spread beyond the orbit by direct invasion of adjacent tissue and hematogenous spread.

Genetics and Cell Biology

The *RB* gene was eventually mapped to chromosome 13q14 (5). Using Southern blot analysis, it was then possible to demonstrate that the second target gene that led to disease was actually the second copy of the *RB* locus, an observation consistent with Knudson’s “two-hit” theory of carcinogenesis (3,6).

The gene, *RB*, consists of 27 exons and encodes a 105-kD nuclear phosphoprotein. pRB plays a central role in the control of cell cycle regulation, particularly in determining transition from G₁ through S (DNA synthesis) phase in virtually all cell types.

In the developing retina, inactivation of the *RB* gene is necessary and sufficient for tumor formation. It is now clear, however, that these tumors develop as a result of a more complex interplay of aberrant expression of other cell cycle control genes. In particular, a tumor surveillance pathway mediated by Arf, MDM2, MDMX, and p53 is activated after loss of pRB during development of the retina. Not only do these observations provide a provocative biologic mechanism for tumor formation in retinoblastoma, but they also point to potential molecular targets for developing novel therapeutic approaches to this tumor.

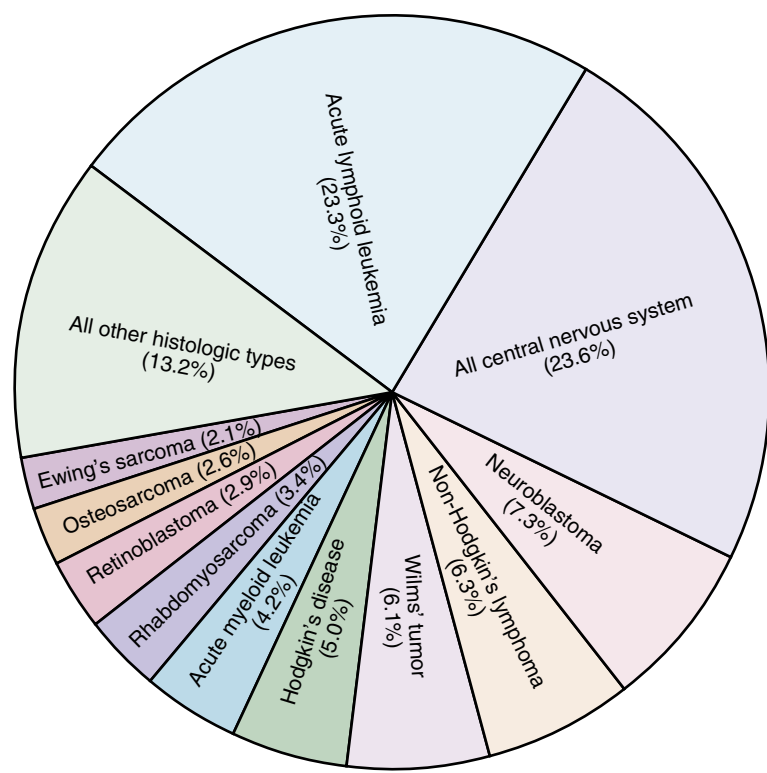


FIGURE 29-1 Relative incidence of childhood neoplasms. (From Ref. 1, with permission.)

Table 29-1 Acquired Molecular and Cytogenetic Abnormalities in Childhood Neoplasms

Solid Tumor	Cytogenetic Abnormality	Genes ^a
Ewing sarcoma	t(11;22)(q24;q12), +8	<i>EWS(22) FLI1(11)</i>
Neuroblastoma	del1p32–36, DMs, HSRs, +17q21-qter	<i>N-MYC</i>
Retinoblastoma	del13q14	<i>Rb</i>
Wilms' tumor	del11p13, t(3;17)	<i>WT1</i>
Synovial sarcoma	t(X;11)(p11;q11)	<i>SSX(X) SYT(18)</i>
Osteogenic sarcoma	del13q14	?
Rhabdomyosarcoma	t(2;13)(q37;q14), t(1;13)(1p36; q14), 3p-, 11p-	<i>PAX3(2) FKHR(13); PAX7(1) FKHR(13)</i>
Peripheral neuroepithelioma	t(11;22)(q24;q12), +8	<i>EWS(22) FLI-1(11)</i>
Astrocytoma	i(17q)	?
Meningioma	delq22	<i>MN1, NF2, ?</i>
Atypical teratoid/ rhabdoid tumor	delq22.11	<i>SNF 5/INI1</i>
Germ cell tumor	i(12p)	?

^a Chromosomal location in parentheses; ?, gene unknown.

Treatment

The treatment of patients with RB depends on the size of the tumor and the extent of tumor invasion at the time of diagnosis. Surgery is the mainstay of treatment for children with unilateral RB. Large intraocular tumors as well those with bilateral multiple tumors are treated with adjuvant chemotherapy.

Wilms' Tumor

Clinical Presentation and Pathology

Wilms' tumor (WT), or nephroblastoma, is an embryonal malignancy that arises from remnants of immature kidney (7). It affects approximately 1 in 7,000 children, usually before the age of 6 years (median age at diagnosis, 3.5 years). Five percent to 10% of children present with synchronous or metachronous bilateral tumors. WT typically presents as an asymptomatic abdominal mass, although a small fraction of children have symptoms such as hematuria or hypertension, which are attributable to the tumor. Approximately 20% of children present with metastatic disease.

The relationship between WT and aberrations of normal development is striking. In early development, the embryonal mesonephros emerges from a complex interaction between epithelial-derived ureteric bud tissue and mesenchymal-derived metanephric blastema through a series of differentiation events. Mature nephrons derived from the mesonephros are composed

of nephroblasts, tubules, and stromal tissues that ultimately form the adult kidney. These different tissues together confer the distinctive “triphasic” histologic features of WT that arise in the intralobar area. Tumors that arise in the perilobar area tend to be biphasic or monomorphic, typically epithelial. This presentation suggests that these tumors arise from a cell that is more prevalent later in development, having a more limited potential to differentiate along multiple lineages.

A peculiar feature of WT is its association with nephrogenic rests, foci of primitive but nonmalignant cells whose persistence suggests a defect in kidney development. These precursor lesions are found within the normal kidney tissue of more than one third of children with WT. Nephrogenic rests may persist, regress spontaneously, or grow into large masses that simulate true WT and present a difficult diagnostic challenge (8). Another intriguing feature of WT is its association with specific congenital abnormalities, including genitourinary anomalies, sporadic aniridia, mental retardation, and hemihypertrophy. A genetic predisposition to WT is observed in two distinct disease syndromes with urogenital system malformations—the WAGR (Wilms’ tumor, aniridia, genitourinary abnormalities, mental retardation) syndrome (9) and the Denys-Drash syndrome (DDS; 9)—and in Beckwith-Wiedemann syndrome (BWS; 10).

Genetics

The WAGR syndrome has been correlated with constitutional deletions of chromosome 11q13 (9). Whereas it is now known that the WAGR deletion encompasses a number of contiguous genes, including the aniridia gene *Pax6*, the cytogenetic observation in patients with WAGR was also important in the cloning of the *WT1* gene at chromosome 11p13. *WT1* spans approximately 50 kb of DNA and contains 10 exons. The *WT1* protein is a transcription factor. The second syndrome closely associated with this locus was initially described by Denys in 1967 and recognized as a syndrome by Drash 3 years later. DDS is a rare association of WT, intersex disorders, and progressive renal failure (10). It has been demonstrated that virtually all patients with DDS carry *WT1* point mutations in the germ line.

WT1 is altered in only 10% of Wilms’ tumors. This observation implies the existence of alternative loci in the etiology of this childhood renal malignancy. One such locus also resides on the short arm of chromosome 11, telomeric of *WT1*, at 11p15. This gene, designated *WT2*, is associated with BWS. Patients with BWS are at increased risk of developing Wilms’ tumor, as well as other embryonic malignancies, including rhabdomyosarcoma (RMS), neuroblastoma, and hepatoblastoma (11). The putative BWS gene maps to chromosome 11p15 (12). Whether the BWS gene and *WT2* are one and the same or two distinct yet closely linked genes remains to be determined. Using long-oligonucleotide array comparative genomic hybridization (array CGH), a novel gene termed *WTX* was identified on chromosome Xq11.1. *WTX* is inactivated in one third of WTs, and tumors with *WTX* mutations lack *WT1* mutations. Bilateral WT or a family history of WT occurs in 1% to 5% of patients.

Although linkage studies have indicated that the gene for familial WT must be distinct from *WT1* and *WT2*, and from the gene that predisposes to BWS, this gene has been neither cytogenetically localized nor isolated.

Treatment

WT was the first of the solid tumors of childhood recognized as being curable even in the setting of metastatic disease. Sequential clinical treatment protocols evaluated by the National Wilms’ Tumor Study have led to effective multimodality approaches that cure up to 90% of children with WT. The cornerstone of therapy is surgery with lack of tumor contamination into the abdominal cavity at the time of tumor removal. Chemotherapy agents are used to treat minimal residual disease and metastatic disease.

Tumors of the Peripheral Nervous System: Neuroblastoma

Clinical Presentation and Pathology

Neuroblastoma (NB) is the most common tumor of the peripheral sympathetic nervous system in children. The embryonic neural crest gives rise to the peripheral nervous system including cranial and spinal sensory ganglia, the autonomic ganglia, the adrenal medulla, and other para-endocrine cells distributed throughout the body.

NB most commonly arise in cells of the adrenal medulla and at other abdominal retroperitoneal sites of the known peripheral nervous system. Approximately 15% of cases occur in the paravertebral thoracic cavity in close association with the dorsal root ganglion. Most cases of NB (60%–70%) present with metastatic disease, most commonly involving bone, bone marrow, and liver. NB is characterized histologically by presence of small, round cells with hyperchromatic nuclei and stippled chromatin. A hallmark of its light microscopic appearance is the presence of Homer-Wright rosettes characterized by tumor cell clusters around a central mesh of cell processes, neuropile.

Genetics and Cell Biology

Nonrandom cytogenetic abnormalities are observed in more than 75% of neuroblastomas. The most common of these is deletion or rearrangement of the short arm of chromosome 1, although loss, gain, and rearrangements of chromosomes 10, 11, 14, 17, and 19 have also been reported. Two other unique cytogenetic rearrangements are highly characteristic of neuroblastoma: homogeneous staining regions and double-minute chromosomes (Figure 29-2). These contain regions of amplification of the *N-myc* gene, an oncogene with considerable homology to the cellular proto-oncogene *c-myc*. *N-myc* amplification is associated with rapid tumor progression and virtually all neuroblastoma tumor cell lines demonstrate amplified and highly expressed *N-myc* (13). Decreased *N-myc* expression is observed in association with the in vitro

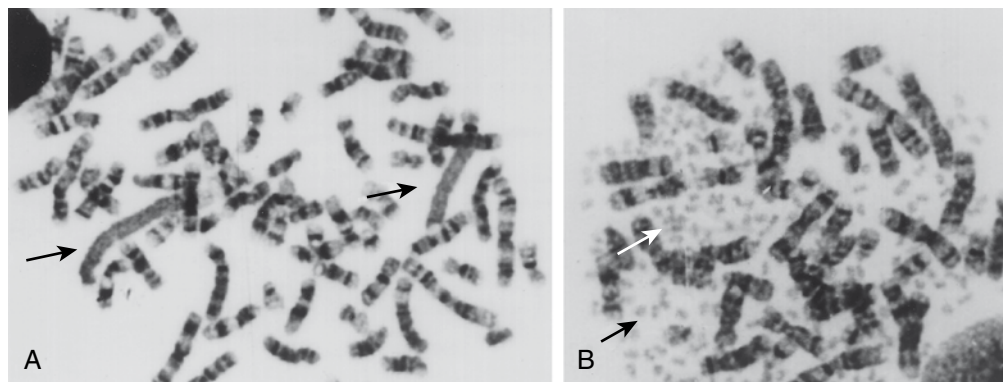


FIGURE 29-2 Homogeneously stained regions and double-minute chromosomes in neuroblastoma.

differentiation of neuroblastoma cell lines (14). This observation formed the basis for therapeutic trials demonstrating a survival advantage to patients treated with *cis*-retinoic acid (15).

Neuroblastoma cells that express the high-affinity nerve growth factor receptor *trkA* can be terminally differentiated by nerve growth factor and demonstrate morphologic changes typical of ganglionic differentiation. Tumors showing ganglionic differentiation and *trkA* gene activation have a favorable prognosis. Expression of *trkB* receptor is associated with poor-prognosis tumors and appears to mediate resistance to chemotherapy.

In addition to chromosomal loss on chromosome 1p36, unbalanced loss of heterozygosity at 11q23 is independently associated with decreased event-free survival. Alterations at 11q23 occur in almost one third of neuroblastomas, being most commonly associated with stage IV disease and age at diagnosis greater than 2.5 years. Telomerase expression and telomere length are yet other valuable markers of clinical significance (16). In particular, short telomere length is predictive of favorable prognosis, regardless of disease stage, whereas long or unchanged telomeres are predictive of poor outcome.

Treatment

The clinical stage of NB disease and age of onset are highly significant independent prognostic variables. For example, a unique presentation of NB, stage IV-S (IV-special) is frequently associated with spontaneous remission (17). This form of the disease typically presents in infants younger than 1 year of age with evidence of remote disease in the liver and bone marrow, though sparing bone. It is not known whether IV-S NB represents metastatic disease or a multifocal nonclonal disorder of neuroblast development. Stages I and II NB can generally be effectively managed with surgical resection alone, although those rare patients with low-stage disease and adverse biologic markers often require adjuvant chemotherapy. Multimodality therapy, including high-dose chemotherapy, hematopoietic stem cell harvest and rescue, and radiation therapy are required to achieve remissions in stage IV (and to a lesser extent stage III) NB, although these are maintained in less than 40% of patients.

Tumors of the Peripheral Nervous System: Ewing Sarcoma and Primitive Neuroectodermal Tumors

Clinical Description and Pathology

The Ewing sarcoma family of tumors (ESFT) is the second most common bone malignancy after osteosarcoma in children and young adults with a peak incidence at age 15. In rare cases, these tumors can arise in the soft tissues. ESFT includes Ewing sarcoma, peripheral primitive neuroectodermal (PPNET), and Askin tumors, among others. These tumors share indistinguishable genetic alterations, immunohistochemical profiles, and lineage-specific marker expression patterns.

Genetics and Cell Biology

PPNET and ES typically carry a t(11;22)(q24;q12) chromosomal rearrangement, although variant translocations have been observed (18). The translocation breakpoint has been molecularly cloned and characterized as an in-frame fusion between the 5' half of the ES gene, *EWS*, on chromosome 22 and the 3' half of the human homologue of an ETS transcription family member, *FLI1*, on chromosome 11. The resultant chimeric protein replaces the DNA-binding domain of *EWS* with the ETS-like binding domain of *Fli-1*, retaining the DNA-binding activity of *Fli-1*. Important transcriptional targets of the *EWS-Fli1* transcription factor may include IGF-I receptor (19), which is thought to play a role in the pathogenesis of ES. Several studies have indicated the importance of the autocrine stimulation of the insulin-like growth factor I receptor (IGF-IR) for cell transformation and proliferation induced by *EWS-Fli1*. Expression profiling analysis has also revealed that *TP53* is transcriptionally up-regulated by the *EWS-ETS* fusion gene. *NKX2.2* has been found to be another target gene of *EWS-Fli1* that is required for malignant transformation. Several variant translocations have also been identified, invariably fusing the *EWS* gene to an ETS family member. Interestingly, it has been suggested that the specific fusion protein expressed in

ESFT has prognostic significance (20). In particular, a rearrangement that joins exon 7 of *EWS* to exon 6 of *Fli1* may confer a more favorable outcome.

Treatment

As in the case of neuroblastoma, therapy for patient with localized PPNET or ES is highly effective, whereas the prognosis for patients with metastatic disease is extremely poor even in the setting of multimodal therapy. Surgery and radiation are used for primary local control with multi-agent chemotherapy, including cyclophosphamide, vincristine, adriamycin, etoposide, and ifosfamide, is used to treat systemic disease.

Rhabdomyosarcoma

Clinical Description and Pathology

Sarcomas arise in supportive tissues that have their origin in embryonic mesenchyme. These tissues include fibrous tissue, muscle, cartilage and bone. Each of the different sarcomas exhibits evidence of differentiation along one or more of these cellular lineages.

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of childhood, with approximately 200 new cases annually in the United States, accounting for nearly 10% of all childhood solid tumors. The incidence of RMS is higher in males than in

females (1.4:1), and most cases are diagnosed in children under the age of 6 (21). Rhabdomyosarcoma is believed to arise from primitive embryonic mesenchymal cells committed to the skeletal muscle lineage; however, RMS tumors have been found in tissues not usually containing striated muscle, such as, commonly, the urinary bladder. Multiple histologic subtypes of RMS exist, including predominantly embryonal (ERMS; 63% of all tumors) and alveolar (ARMS; 19%) morphologies. The alveolar subtypes tend to occur in the extremities and exhibit a more aggressive clinical behavior than their ERMS counterparts, which tend to present in an axial distribution and with a somewhat more favorable prognosis. Although the overall survival for childhood RMS is $\approx 75\%$, those with metastatic disease have a less than 20% chance of cure.

Genetics and Cell Biology

Characteristic genetic lesions have been found in both major subtypes of RMS. More than 75% of tumors of alveolar subtype demonstrates one of two chromosomal translocations, $t(2;13)$ (q35;q14) or $t(1;13)(p36;q14)$ (22,23), which fuse the 5' DNA-binding region of *PAX-3* on chromosome 2 or *PAX-7* on chromosome 1, respectively, that are implicated in neuromuscular development, to the 3' transactivation domain region of *FKHR* (*FOXO1A*) gene a member of the forkhead family of transcription factors commonly associated with regulation of apoptosis (Figure 29-3). Tumors with the $t(2;13)$ translocation have a much poorer prognosis than those with the rarer $t(1;13)$ rearrangement.

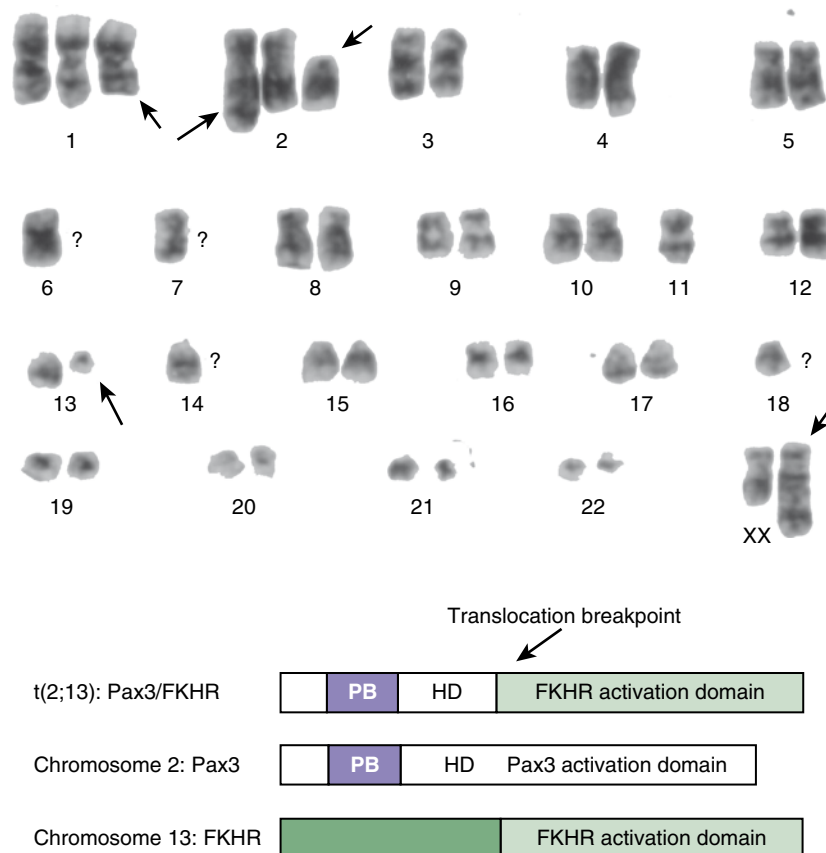


FIGURE 29-3 Translocation breakpoints in rhabdomyosarcoma.

Additional epigenetic or genetic events seem required for RMS tumorigenesis. PAX-3–FKHR fusion is associated with increased expression of *c-met*. Met is the receptor tyrosine kinase for hepatocyte growth factor/scatter factor and is overexpressed in embryonal and alveolar RMS. Other frequently reported genetic alterations that may be common to embryonal and alveolar RMS include activated forms of N- and K-RAS, inactivating *TP53* mutations, and amplification and overexpression of *MDM2*, *CDK4*, and *N-MYC*.

The genetics of ERMS tumors are distinct from ARMS primarily in that they display a specific loss of heterozygosity (LOH) at chromosome 11p15, involving the loss of maternal and duplication of paternal genetic material. Classically associated with this LOH is the overexpression, due to a failure of imprinting, of the insulin-like growth factor 2 (*IGF-2*), which may be involved in the tumorigenic cascade that leads to ERMS. Other molecular alterations present in RMS include dysregulation of *TP53* and *RB* tumor suppressors, as well as the potent oncogene *RAS*.

At the molecular level, embryonal tumors are characterized by LOH at the 11p15 locus, which is of particular interest because this region harbors the *IGF2* gene (24). The LOH at 11p15 occurs by loss of maternal and duplication of paternal chromosomal material (25). Although LOH is normally associated with loss of tumor suppressor gene activity, in this instance LOH with paternal duplication may result in activation of *IGF2*. This occurs because *IGF2* is now known to be normally imprinted; that is, this gene is normally transcriptionally silent at the maternal allele, with only the paternal allele being transcriptionally active. Thus, LOH with paternal duplication potentially leads to a twofold gene-dosage effect of the *IGF2* locus.

In addition to the somatic molecular changes associated with RMS, the tumor is also frequently observed in Li-Fraumeni syndrome (see following section) in which carriers harbor constitutional mutations of the *TP53* tumor suppressor gene. The possible importance of the patched gene, *PTCH*, in the development of RMS is suggested by the finding that mice lacking this gene develop RMS. *PTCH* is mutated in the germ line of patients with Gorlin syndrome, a disorder that includes predisposition to tumor (medulloblastoma) development. Strikingly, *PTCH* is shown to regulate another gene, *GLI*, which is found to be amplified in RMS and Gorlin syndrome–associated tumors.

Treatment

The management of these tumors typically includes local control with both surgery and radiation treatment, with neo-adjuvant chemotherapy being used for control of known and micrometastatic disease. It is notoriously difficult to achieve a sustained remission, or cure, for children with metastatic RMS, particularly those with the alveolar variant.

Childhood Sarcomas: Osteosarcoma

Clinical Description and Pathology

Osteosarcoma (OS) occurs most frequently during adolescence during a period of rapid bone growth. It is the most common

tumor in this age group other than those of hematopoietic tissues. OS most commonly occurs at metaphyseal growth plates of long bones, develops earlier in girls than in boys, and is more frequent in taller children. These observations suggest an important role for cellular proliferation in the oncogenic conversion of immature bone precursors from which these tumors are thought to arise. The histologic diagnosis of OS is made when tumor osteoid and disorganized bone can be identified within malignant stromal tissues. A wide range of histologic patterns is seen, although the natural history of these variants is not yet clinically distinguishable. Tumors are classified as osteoblastic, chondroblastic, or fibroblastic OS depending on whether the predominant differentiation is a long bone, cartilage, or stromal tissue pathway, respectively.

Genetics and Cell Biology

OS is characterized typically by presence of complex unbalanced karyotypes and alterations of the *p53* and retinoblastoma pathways in most cases (26). Combined inactivation of the *RB1* and *TP53* tumor suppressor pathways are observed in most OS, indicating important roles for both these genes in OS pathogenesis. Further evidence for the role of *p53* in OS pathogenesis includes the predisposition of patients with germ-line *TP53* mutations (Li-Fraumeni syndrome) to develop OS. There is low prognostic significance of *TP53* mutations in sporadic OS, with no impact on distant recurrence. Furthermore, *p53* status is concordant in paired samples of primary and distant metastases, suggesting *p53* pathway alterations may occur early in OS pathogenesis. Modifying effects of other expressed genes are being explored in OS. In particular, amplification of chromosome 12q13 region (containing *MDM2* and *CDK4*) or deletion of *INK4A* can disrupt both the *p53* and *RB* pathways. Many recurrent, nonrandom chromosomal abnormalities are observed in OS. Common numerical abnormalities in OS include gain of chromosome 1; loss of chromosomes 9, 10, 13 (at the *RB1* locus), and/or 17 (at the *TP53* locus); and partial or complete loss of the long arm of chromosome 6. Frequent structural abnormalities include rearrangements of chromosomes 11, 19, and 20. Genome-wide efforts to identify potential tumor suppressor genes associated with the loss of heterozygosity (LOH) in OS are under way. Examination of 38 chromosomal arms from OS tumor samples for LOH has found that the mean frequency of LOH is 30.79% for any chromosome arm, an unusually high mean frequency for a childhood tumor. Moreover, several chromosome arms (3q, 13q, 17p, and 18q) underwent LOH with a frequency more than two standard deviations higher than the average ($p < 0.002$; 26). Further mitotic mapping has identified minimal regions thought to contain candidate tumor suppressor loci on chromosomal arms 3q26.2 and 18q21.33 (27).

Abnormalities of bone growth and remodeling are thought to play an important role in the pathogenesis of OS. Normal bone repair involves proliferation of mesenchymally derived precursor cells mediated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), IGFs, interferon- α , and other mitogens. The PDGF receptor encodes a tyrosine kinase, and when PDGF binds to its receptor it induces expression of *fos*, *myc*, and a cascade of cellular genes important for initiating proliferation. EGF may also play a role in OS pathogenesis. Some

OS cell lines express EGF receptor and proliferate in response to exposure to EGF. The mitogenic response to both EGF and PDGF may be blocked by TGF- β , which at low doses, may stimulate proliferation of cells.

Interest in other signaling pathways has implicated the Fas cell death pathway in determining chemosensitivity and metastatic behavior in OS (28). Tumor cells expressing surface Fas will apoptose when Fas ligand (FasL) is present unless a mechanism of resistance is present. This has been suggested by studies in which metastasis-prone OS cell lines when transfected with Fas demonstrate reduced metastatic potential.

Treatment

Surgery is the primary therapeutic modality in the management of osteosarcoma. Neo-adjuvant chemotherapy is used to control micrometastases, which are present in 75% of patients. The response to the chemotherapy, as measured by histologic grading of degree of tumor necrosis, is a key prognostic factor. Biologic response modifiers, monoclonal antibodies and targeted small-molecule kinase inhibitors have not impacted treatment of osteosarcoma.

Cancer Predisposition Syndromes

Several hereditary cancer syndromes are associated with the occurrence of childhood as well as adult-onset neoplasms. Although it is beyond the scope of this chapter to describe them all, it is worthwhile to discuss a few to highlight the important molecular basis on which these disorders develop (Table 29-2).

Li-Fraumeni Syndrome

Li-Fraumeni familial cancer syndrome (LFS) is the prototypical familial cancer predisposition syndrome. The definition of classical LFS requires a proband with a sarcoma diagnosed before 45 years of age, a first-degree relative diagnosed as having any cancer when younger than 45 years, and a first- or second degree relative with a diagnosis of cancer when younger than 45 years or a sarcoma at any age (29). The classic spectrum of tumors that includes soft-tissue sarcomas, osteosarcomas, breast cancer, brain tumors, leukemia, and adrenocortical carcinoma (ACC) has been overwhelmingly substantiated by numerous subsequent studies, although other cancers, usually of particularly early age of onset, are also observed. Similar patterns of cancer that do not meet the classic definition have been coined Li-Fraumeni-like syndrome (LFS-L).

Germ-line alterations of the *TP53* tumor suppressor gene are associated with LFS (30,31). These are primarily missense mutations that yield a stabilized mutant protein. The spectrum of germ-line *TP53* mutations is similar to that of somatic mutations found in a wide variety of tumors. Carriers are heterozygous for the mutation, and in tumors derived from these individuals, the second (wild-type) allele is frequently deleted or mutated, leading to functional inactivation. Several comprehensive databases document all reported germ-line (and somatic) *TP53* mutations and are of particular value in evaluating novel mutations as well as phenotype-genotype correlations. Approximately 75% of "classic" LFS families have detectable *TP53* alterations. It is not clear whether the remainder are associated with the presence of modifier genes, promoter defects yielding abnormalities of p53 expression, or simply the result of weak phenotype-genotype correlations.

Table 29-2 Hereditary Syndromes Associated with Childhood Neoplasms

Syndrome	OMIM Entry	Major Tumor Types	Mode of Inheritance	Genes
Hereditary gastrointestinal malignancies				
Adenomatous polyposis of the colon	175100	Colon, thyroid, stomach, intestine, hepatoblastoma	Dominant	<i>APC</i>
Juvenile polyposis	174900	Gastrointestinal	Dominant	<i>SMAD4/DPC4</i>
Peutz-Jeghers syndrome	175200	Intestinal, ovarian, pancreatic	Dominant	<i>STK11</i>
Genodermatoses with cancer predisposition				
Nevoid basal cell carcinoma syndrome	109400	Skin, medulloblastoma	Dominant	<i>PTCH</i>
Neurofibromatosis type 1	162200	Neurofibroma, optic pathway glioma, peripheral nerve sheath tumor	Dominant	<i>NF1</i>
Neurofibromatosis type 2	101000	Vestibular schwannoma	Dominant	<i>NF2</i>
Tuberous sclerosis	191100	Hamartoma, renal angiomyolipoma, renal cell carcinoma	Dominant	<i>TSC1/TSC2</i>
Xeroderma pigmentosum	278730, 278700, 278720, 278760, 278740, 278780, 278750, 133510	Skin, melanoma, leukemia	Recessive	<i>XPA,B,C,D,E,F,G, POLH</i>

(Continued)

Table 29-2 Hereditary Syndromes Associated with Childhood Neoplasms—(Continued)

Syndrome	OMIM Entry	Major Tumor Types	Mode of Inheritance	Genes
Rothmund-Thomson syndrome	268400	Skin, bone	Recessive	<i>RECQL4</i>
Leukemia/lymphoma predisposition syndromes				
Bloom syndrome	210900	Leukemia, lymphoma, skin	Recessive	<i>BLM</i>
Fanconi anemia	227650	Leukemia, squamous cell carcinoma, gynaecological system	Recessive	<i>FANCA,B,C,D₂,E,F,G</i>
Schwachman Diamond syndrome	260400	Leukemia/myelodysplasia	Recessive	<i>SBDS</i>
Nijmegen breakage syndrome	251260	Lymphoma, medulloblastoma, glioma	Recessive	<i>NBS1</i>
Ataxia teleangiectasia	208900	Leukemia, lymphoma	Recessive	<i>ATM</i>
Genitourinary cancer predisposition syndromes				
Simpson-Golabi-Behmel syndrome	312870	Embryonal tumors, Wilms' tumor	X-linked	<i>GPC3</i>
von Hippel-Lindau syndrome	193300	Retinal and central nervous hemangioblastoma, pheochromocytoma, renal cell carcinoma	Dominant	<i>VHL</i>
Beckwith-Wiedemann syndrome	130650	Wilms' tumor, hepatoblastoma, adrenal carcinoma, rhabdomyosarcoma	Dominant	<i>CDKN1C/NSD1</i>
Wilms' tumor syndrome	194070	Wilms' tumor	Dominant	<i>WT1</i>
WAGR syndrome	194072	Wilms' tumor, gonadoblastoma	Dominant	<i>WT1</i>
Costello syndrome	218040	Neuroblastoma, rhabdomyosarcoma, bladder carcinoma	Dominant	<i>H-Ras</i>
Central nervous system predisposition syndromes				
Retinoblastoma	180200	Retinoblastoma, osteosarcoma	Dominant	<i>RB1</i>
Rhabdoid predisposition syndrome	601607	Rhabdoid tumor, medulloblastoma, choroidplexus tumor		<i>SNF5/INI1</i>
Medulloblastoma predisposition	607035	Medulloblastoma	Dominant	<i>SUFU</i>
Sarcoma/bone cancer predisposition syndromes				
Li-Fraumeni syndrome	151623	Soft-tissue sarcoma, osteosarcoma, breast, adrenocortical carcinoma, leukemia, brain tumor	Dominant	<i>TP53</i>
Multiple exostosis	133700, 133701	Chondrosarcoma	Dominant	<i>EXT1/EXT2</i>
Werner syndrome	277700	Osteosarcoma, meningioma	Recessive	<i>WRN</i>
Endocrine cancer predisposition syndromes				
MEN1	131000	Pancreatic islet cell tumor, pituitary adenoma, parathyroid adenoma	Dominant	<i>MEN1</i>
MEN2	171400	Medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia	Dominant	<i>RET</i>

OMIM, Online Mendelian Inheritance in Man.

(i.e., the broad clinical definition encompasses families that are not actual members of LFS). The variability in type of cancer and age of onset within and between LFS families suggests that expression of modifier genes might influence the underlying mutant *TP53* genotype.

Beckwith-Wiedemann Syndrome

Beckwith-Wiedemann syndrome (BWS) occurs with a frequency of 1 in 13,700 births. BWS is associated with a wide spectrum of phenotypic stigmata including hemihypertrophy/hyperplasia, exomphalos, macroglossia, gigantism, and ear pits (posterior aspect of the pinna). Laboratory findings may include profound neonatal hypoglycemia (extremely common), polycythemia, hypocalcemia, hypertriglyceridemia, hypercholesterolemia, and high serum α -fetoprotein levels (11). With increasing age, phenotypic and laboratory features of BWS become less pronounced. Although neurocognitive defects are not universal in BWS, early diagnosis of the condition is crucial to avoid deleterious neurologic effects of neonatal hypoglycemia and to initiate an appropriate screening protocol for tumor development. The increased risk for tumor formation in BWS patients is estimated at 7.5% and is further increased to 10% if hemihyperplasia is present. Tumors occurring with the highest frequency include Wilms' tumor, hepatoblastoma, neuroblastoma, rhabdomyosarcoma, and ACC.

The genetic basis of BWS is complex, and it does not appear that a single gene is responsible for the BWS phenotype. Various 11p15 chromosomal or molecular alterations have been associated with the BWS phenotype (Table 29-3) and its tumors (32). Abnormalities in this region impact an imprinted domain, indicating that it is more likely that normal gene regulation in this part of chromosome 11p15 occurs in a regional manner and may depend on various interdependent factors or genes. These include the paternally expressed genes *IGF2* and *KCNQ10T1* and the maternally expressed genes *H19*, *CDKN1C*, and *KCNQ1*. Paternal uniparental disomy, in which two alleles are inherited from one parent (the father), has been

reported in approximately 15% of sporadic BWS patients (33). It is interesting that the insulin/IGF2 region is always represented in the uniparental disomy, although the extent of chromosomal involvement is highly variable. Alterations in allele-specific DNA methylation of *IGF2* and *H19* reflect this paternal imprinting phenomenon (33).

Gorlin Syndrome

Nevoid basal cell carcinoma syndrome, or Gorlin syndrome, is a rare autosomal dominant disorder characterized by multiple basal cell carcinomas, developmental defects including bifid ribs and other spine and rib abnormalities, palmar and plantar pits, odontogenic keratocysts, and generalized overgrowth (34). The sonic hedgehog (SHH) signaling pathway directs embryonic development of a spectrum of organisms. Gorlin syndrome appears to be caused by germ-line mutations of the tumor suppressor gene *PTCH*, a receptor for SHH. Medulloblastoma develops in approximately 5% of patients with Gorlin syndrome. Furthermore, approximately 10% of patients diagnosed with medulloblastoma by the age of 2 years are found to have other phenotypic features consistent with Gorlin syndrome and harbor germ-line *PTCH* mutations (35). Although Gorlin syndrome develops in individuals with germ-line mutations of *PTCH*, a subset of children with medulloblastoma harbor germ-line mutations of another gene, *SUFU*, in the SHH pathway, with accompanying LOH in the tumors.

Multiple Endocrine Neoplasia

The multiple endocrine neoplasia (MEN) disorders comprise at least three different diseases, MEN type 1, MEN type 2A, and MEN type 2B, which are all cancer predisposition syndromes that affect different endocrine organs. The most common features of MEN type 1 are parathyroid adenomas (about 90% of the cases), pancreatic islet cell tumors (50%–75% of the cases), and pituitary adenomas (25%–65% of the cases; 36). MEN2A is associated with medullary thyroid carcinoma, parathyroid adenoma, and

Table 29-3 Beckwith-Wiedemann Syndrome Genetic and Epigenetic Subgroups

	DNA	RNA	Karyotype	Frequency	Inheritance
A. Regional	Paternal 11p15 UPD Disruption of <i>KCNQ10T1</i>		Normal 11p15 Duplication 11p15 Transl/Inver	10%–20% 1% 1%	Sporadic Sporadic Sporadic
B. Domain 1	<i>H19</i> hypermethylation Normal <i>H19</i> methylation	<i>IGF2</i> LOI <i>IGF2</i> LOI	Normal Normal	2% 25%–50%	Sporadic Sporadic
C. Domain 2	<i>CDKN1C</i> mutation <i>CDKN1C</i> mutation <i>KvDMR1</i> LOM	KNQ10T1 LOI	Normal Normal Normal	5%–10% 25% 50%	Sporadic AD Sporadic
D. Other	Unknown Unknown	Unknown	Normal Normal	5% 10%–20%	AD Sporadic

AD, autosomal dominant; LOI, loss of imprinting; LOM, loss of methylation; UPD, uniparental disomy.

pheochromocytoma. MEN2B is a related disorder, but with onset of the tumors in early infancy, ganglioneurinoma of the gastrointestinal tract, and skeletal abnormalities.

Although MEN1 is caused by mutation in the tumor-suppressor gene, *MEN1*, MEN2A and 2B are caused by mutations in the proto-oncogene *RET*. Further studies confirmed constitutional mutations in the *RET* proto-oncogene in families with MEN2A and 2B. The pattern of mutations seen in MEN2 families does not follow the "two-hit hypothesis" for tumor suppressor genes: The *RET* proto-oncogene is not inactivated, and there is no loss of the second allele in the tumors. Thus the predisposition to cancer in families with MEN2 is based on the inheritance of an activating mutation in the *RET* proto-oncogene. Genetic testing is possible by direct mutation analysis of the ten exons of the gene.

Molecular and Clinical Surveillance for Cancer Predisposition in Children

As in tumors of adults, continued investigation of the molecular alterations that underlie tumor pathogenesis in children can be expected to provide insights that will lead to highly specific molecular therapeutic target and that in turn should lead to more specific, more efficacious, and less toxic therapies. Because tumors associated with highly penetrant genetic predispositions often

occur early in life, such insights into pathogenesis also provide novel opportunities for the diagnosis of cancer in children. The evidence to justify the use and continued refinement of molecular analysis for tumor diagnosis, prognosis, and development of novel therapeutic avenues for children with cancer is overwhelming. The use of molecular screening as a tool for identification of children at risk for the purpose of developing rational clinical surveillance guidelines is more controversial. Several issues are worth noting. Based on recommendations of the American Society of Clinical Oncology (37), genetic testing should only be undertaken with fully informed consent, including elements of risk assessment, psychological implications of test results (both benefits and risks), risks of employer or insurance discrimination, confidentiality issues, and options and limitations of medical surveillance or prevention strategies. When children are not competent to give informed consent, the main consideration should be for the welfare of the child. Although screening for some mutations, such as *RET* or *RB*, are associated with clearly defined beneficial medical management decisions that lead to improved outcomes, it has been argued that presymptomatic identification of other gene mutations, such as in Li-Fraumeni syndrome, are of less obvious clinical benefit. Regardless of the scenario, the complexity of the issues underlies the importance that referral of these patients and families is made to an experienced multidisciplinary team including oncologists, geneticists, psychologists, and genetic counselors to facilitate the most appropriate management.

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Molecular Basis of Lung Cancer

Accumulating evidence indicates that molecular abnormalities drive the malignant phenotype of lung cancer cells, contribute to therapeutic resistance, and present tumor-specific targets for therapy. Thus, understanding the molecular genetics of lung cancer will be central to developing new diagnostic and therapeutic approaches. Molecular genetic studies of lung cancer pathogenesis have revealed that clinically overt lung cancers harbor multiple genetic and epigenetic alterations numbering more than 20 per individual tumor. Additionally, many of these abnormalities can be found in preneoplastic lesions and histologically normal bronchial epithelial cells, supporting the notion that human lung cancer develops from normal epithelial cells through a multistep process involving successive genetic and epigenetic abnormalities, usually caused by cigarette smoking. These abnormalities contribute to the initiation, development, and maintenance of lung cancer. These molecular findings are being translated into the clinical setting to provide novel approaches for prevention, early detection, and treatment of lung cancer with promising results (Table 30-1 and Figure 30-1).

Etiology of Lung Cancer

Tobacco Smoke and Lung Cancer

Tobacco smoke is the major cause of lung cancer, and smokers are 14-fold more likely to develop lung cancer than nonsmokers. Especially important, tobacco smoke carcinogens are the polycyclic aromatic hydrocarbons (PAHs) and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which play major roles by inducing DNA adduct formation. It has been postulated that genetic polymorphisms in *P450* and *CYP* family genes by altering PAH and NNK metabolism may predispose people to develop lung cancer. Thus, many studies have examined the relationship between the polymorphic variants of *P450* and *GST* genes and the risk for lung cancer; however, no polymorphism predicting lung cancer risk has yet been brought to clinical use.

Inherited Susceptibility to Lung Cancer

Identifying genes that determine inherited susceptibility of individuals to lung cancer would provide important information

to identify persons at highest risk of developing lung cancer. Epidemiologic studies show a 2.5-fold increased risk attributable to a family history of lung cancer after controlling for tobacco smoke, suggesting that genetic factors other than those related to metabolizing carcinogens from tobacco smoke may influence a person's susceptibility to lung cancer. A recent large-scale linkage analysis (52 pedigrees) by the Genetic Epidemiology of Lung Cancer Consortium (GELCC) suggests that a major autosomal susceptibility locus for inherited lung cancer exists on 6q23–25 (146cM to 164cM; 1). The identification of a gene in this region whose mutated form predisposes to lung cancer will be immediately used to screen populations to identify persons having the mutant allele so that these people can be entered into early detection and prevention programs.

Virus Infection and Lung Cancer

The incidence of adenocarcinoma is increasing worldwide, especially in nonsmoking women, and thus unknown factors other than tobacco smoke that preferentially predispose women to lung cancer may exist. One such factor is human papillomavirus (HPV) infection with HPV E6 inactivating p53 and HPV E7 inactivating retinoblastoma (RB) proteins. A summary of studies analyzing 2,468 lung cancer cases reported the overall rate of HPV infection in lung cancer to be 22% (2). Higher frequencies (70%–80%) of HPV infection were reported by Asian and some northern European countries, whereas low frequencies (<5%) were reported from the United States, suggesting that geographic and/or ethnic factors determining the susceptibility of persons to HPV infection may exist. Importantly, one Taiwanese study reported that HPV-16/18 infection is a significant risk factor for developing lung adenocarcinoma in nonsmoking Taiwanese women, suggesting its contribution to lung cancer pathogenesis (2).

Jaagsiekte sheep retrovirus (JSRV) may be involved in the pathogenesis of human lung cancer. JSRV causes contagious lung adenocarcinoma in sheep, known as ovine pulmonary adenocarcinoma (OPA), which is very similar to human bronchioloalveolar carcinoma (BAC), a rare subtype of adenocarcinoma. JSRV causes oncogenesis by its native envelope protein, JSRV Env. However, studies using polymerase chain reaction (PCR) and/or immunohistochemistry analysis have failed to detect JSRV virus in human lung cancer.

Table 30-1 Genetic Alterations Found in Lung Cancer and Drugs or Therapeutics Targeting these Alterations

Gene	Type of Alteration	Drugs or Therapeutics Targeting Alterations
<i>EGFR</i>	Mutation and amplification	Tyrosine kinase inhibitors (gefitinib, erlotinib) Chimeric IgG monoclonal antibody (cetuximab)
<i>HER2</i>	Mutation and amplification	Pan-ERBB tyrosine kinase inhibitor (canertinib) Humanized monoclonal antibody (trastuzumab)
<i>c-KIT</i>	Overexpressed	Tyrosine kinase inhibitor (imatinib)
<i>SRC</i>	Constitutively activated	Src inhibitor (dasatinib)
<i>BRAF</i>	Mutation	Raf kinase inhibitor (sorafenib)
<i>RAS</i>	Mutation	Farnesyltransferase inhibitors (tipifarnib, lonafarnib)
<i>MEK</i>	Constitutively activated	Inhibitor of MEK (CI-1040, PD325901).
<i>PI3K/Akt/mTOR</i>	Constitutively activated	PI3K inhibitor (LY294002) mTOR (rapamycin) and its derivatives (CCI-779, RAD001, AP23576)
<i>BCL2</i>	Overexpressed	Antisense oligonucleotide (oblimersen sodium) Inhibitor of BCL-2 (ABT-737)
<i>p53</i>	Mutation and deletion	<i>p53</i> adenoviral vector (Advexin)
<i>FUS1</i>	Loss of protein expression	<i>FUS1</i> nanoparticle (DOTAP:Chol- <i>FUS1</i>)
<i>VEGF</i>	Overexpressed	Humanized monoclonal antibody (bevacizumab) VEGFR-2 and EGFR inhibitor (ZD6474)
<i>Telomerase</i>	Overexpressed	Telomerase template antagonist (GRN163L)

From Sato et al. A translational view of the molecular pathogenesis of lung cancer. Journal of thoracic oncology 2007 (50). Abbreviations: EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

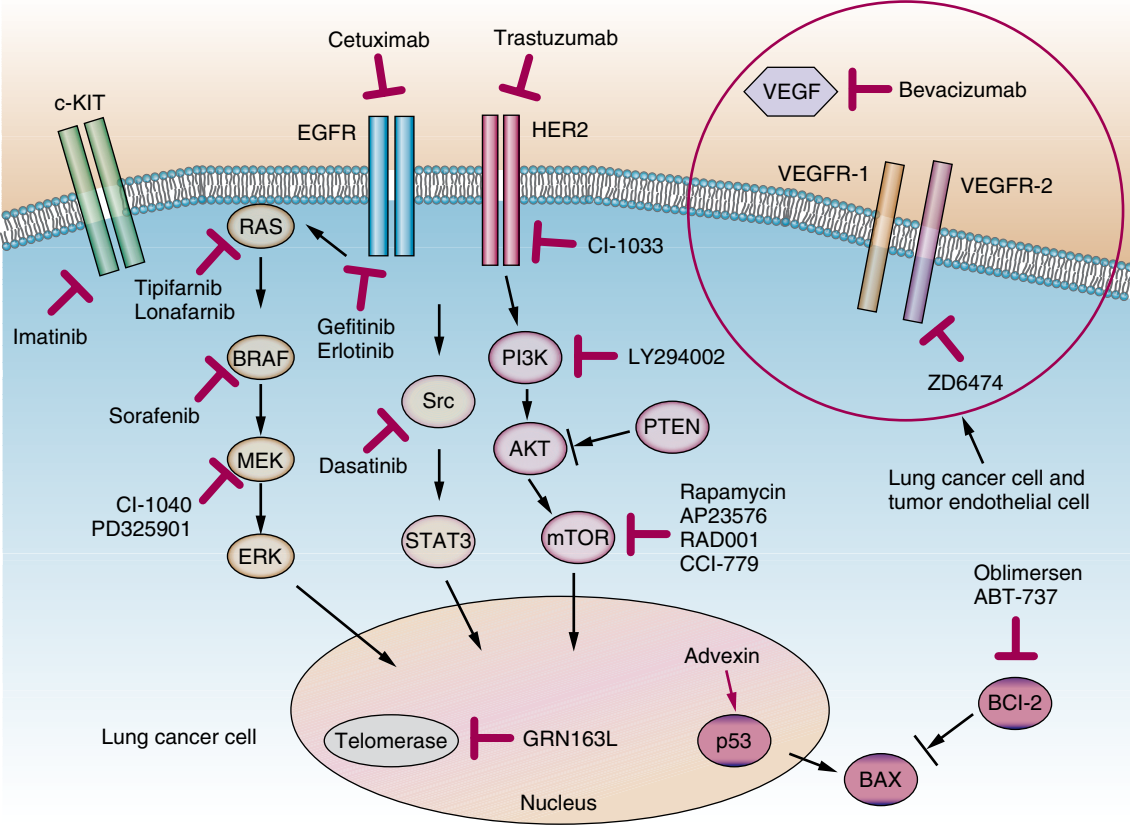


FIGURE 30-1 TARGETED THERAPY OF LUNG CANCER. The proteins in signal transduction cascades that are frequently affected in lung cancer are represented by different color ovals. Proteins in the same cascade are colored similarly. The drugs that are under development or that target these proteins are represented at each point. Proteins in the red circle are expressed in both lung cancer cells and endothelial cells in the tumor vasculature. EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Genomic Instability in Lung Cancer

Aneuploidy is a common property of lung cancers, and persistent chromosomal instability is found in human lung cancer with significant aneuploidy (3). Although mitotic checkpoint genes, such as *BUB1* and *MAD1*, are only rarely mutated in lung cancer, the mitotic checkpoint is frequently (44%) impaired in non-small cell lung cancers (NSCLCs; 4). DNA microsatellite instability (MSI), defined as a DNA sequence change of any length due to insertion or deletion of repeating units in tumor microsatellite DNA sequence compared with DNA from normal tissues, occurs in 35% of small cell lung cancers (SCLCs) and 22% of NSCLCs, with a wide range of MSI frequencies in 13 studies (5). Nevertheless, whether MSI in lung cancer results in alteration of critical genes involved in pathogenesis of lung cancer remains to be elucidated. Finally, several studies show that DNA with MSI could be detectable in blood from patients with lung cancer, suggesting its usefulness as a tool for diagnosis and early detection for lung cancer.

Abnormalities in Growth-Signaling Pathways (Activation of Oncogenes)

Activation of oncogenes confers increased proliferation ability on cancer cells as well as an influence on differentiation, senescence, and apoptosis. Molecular analysis of lung cancer revealed genetic alterations of several oncogenes, including *RAS*, epidermal growth factor receptor (*EGFR*), and *MYC*. The concept of “oncogene addiction” has been proposed (6). This represents a cellular physiologic state where the tumor cells depend on continued activity of specifically activated or overexpressed oncogene to maintain their malignant phenotype. This has important ramifications in the clinic. If the function of such a gene product can be removed by targeted therapy, the therapy could selectively eliminate cancer cells. An excellent example of this concept for lung cancer is *EGFR* tyrosine kinase (TK) domain mutations. Lung cancer cells with mutant *EGFR*s show exquisite sensitivity to tyrosine kinase inhibitors (TKIs; 7,8). Genome-wide single-nucleotide polymorphism (SNP) array analysis and sequencing screens are being performed to find additional oncogenes that are mutated or amplified and thus could be “druggable” targets.

Receptor Tyrosine Kinases

EGFR Family

The *EGFR* family of receptors are transmembrane TK receptors and are composed of *EGFR* (*HER1* or *ERBB1*), *HER2* (*EGFR2* or *ERBB2/NEU*), *HER3* (*EGFR3* or *ERBB3*), and *HER4* (*EGFR4* or *ERBB4*). Upon ligand binding, these *EGFR* family members form active homo- and heterodimers, leading to autophosphorylation and activation of intracellular signaling cascades. Both *EGFR* ($\approx 70\%$) and *HER2* ($\approx 30\%$) are frequently overexpressed in NSCLCs whereas they are rarely overexpressed in SCLCs (5). Drugs targeting these receptors include small molecules that target

TK domain (preventing adenosine triphosphate [ATP] from binding to the domain) and antibodies that target the extracellular domain and thus compete with ligand binding to the receptor. Drugs targeting *EGFR* or *HER2* include the small-molecule TKIs, gefitinib (Iressa), erlotinib (Tarceva) and the monoclonal antibodies, cetuximab (Erbix targeting *EGFR*), and trastuzumab (Herceptin targeting *HER2*).

Several mutations in the TK domain of *EGFR* have been described in lung cancer (7,8). Importantly, these mutations correlate with the tumor drug sensitivity to TKIs (7,8). *EGFR* mutations occur in 24% of $\approx 2,000$ unselected NSCLC samples (9). The *EGFR* mutations occur exclusively in the first four exons (18–21 exons) of TK domain and are categorized into three different types (deletions [44%], missense point mutations [41%], and insertions; (9). An intriguing characteristic of *EGFR* mutations is that they often occur in highly selected patient populations: adenocarcinoma histologies, never-smokers, East Asians, and women (9). A “second” TK domain mutation (T790M) was reported in the same tumors that have the *EGFR* TK domain mutations (10). This mutation was found in patients who were resistant to TKIs or relapsed after TKI treatment, suggesting its contribution to acquired resistance to TKIs. However, it should be noted that this mutation is also found in patients who have not been treated with TKI. In addition, the discovery of a family with an *EGFR* T790M mutation in the germ line associated with familial NSCLC suggests that this mutation could predispose people to lung cancer. There are other TKIs (e.g., HKI-272) that inhibit *EGFR* with the T790M mutation, and derivatives of these are being developed for clinical use.

In addition to the mutation status of *EGFR*, several other predictive markers correlate with TKI response, including high-level amplification of *EGFR*, elevated *EGFR* protein, amplification of *HER2*, and activation of Akt. Recently, *c-Met* amplification has been associated with TKI resistance. These findings have prompted the design of clinical trials that incorporate the patient’s clinicopathologic data and molecular biological features (*EGFR* mutation and/or amplification) of the tumors. These studies are expected to maximize the benefit of TKI treatment for NSCLC.

Several studies have shown oncogenic property of mutant *EGFR*s found in lung cancers. Retrovirus introduction of mutant *EGFR* confers anchorage-independent growth ability to immortalized human bronchial epithelial cells (11). Moreover, transgenic mice harboring the point or the deletion mutation of *EGFR* develop lung adenocarcinomas with very similar histology to those seen in patients with *EGFR* mutations (12,13). Notably, the removal of mutant *EGFR* functions in such mouse models results in regression of the adenocarcinoma, indicating that continued mutant *EGFR* function is necessary to maintain tumors. These results suggest that mutant *EGFR* is required for initiation and maintenance of the tumors. How mutant *EGFR* exerts its oncogenic ability remains to be fully elucidated. However, studies have shown that mutant *EGFR* is constitutively activated, has enhanced sensitivity to ligand-dependent activation, and selectively transduces survival signals (Akt and STATs).

HER2 mutations occur in approximately 2% (16/791) of NSCLCs (14). All reported *HER2* mutations are in-frame insertions in exon 20 and target the corresponding TK domain region as in *EGFR* insertion mutations. Interestingly, these mutations frequently occur in the same subpopulations as those with *EGFR*

mutations (adenocarcinomas, never-smokers, East Asians, and women; 14). Whether tumors with *HER2* mutations are sensitive to trastuzumab remains to be demonstrated.

Finally, lung cancers with *EGFR* mutations are more sensitive to ionizing radiation than those without *EGFR* mutations, which potentially provides a molecular basis for combined modality treatment involving TKIs and radiotherapy (15).

c-KIT

SCLC but not NSCLC frequently express (40%–70%) both the receptor c-KIT and its ligand, stem cell factor (SCF). However, unlike gastrointestinal stromal tumors, activating c-KIT mutations in lung cancers are rare, and two clinical studies and a mouse xenograft study failed to show tumor regression in SCLC by monotherapy with the drug imatinib (Gleevec), which inhibits c-KIT activity.

The RAS/RAF/MEK/ERK Pathway

Activating oncogenic mutations in the *RAS* genes (usually *KRAS*) are found in several human cancers, including lung cancer. *RAS* mutations occur in 10%–15% of NSCLCs, especially in adenocarcinoma (20%–30%), but almost never in SCLCs (5). The mutations involve codons 12, 13, and 61, all of which influence intrinsic GTPase activity. Approximately, 70% of *KRAS* mutations are G-T transversions, which are associated with PAH and NNK in tobacco smoke. Oncogenic *KRAS* (e.g., *KRAS*^{V12} mutant) activates cell signaling pathways important to cellular transformation, and thus oncogenic *KRAS* represents an important therapeutic target. A number of drugs that target different aspect of *RAS* function and metabolism have been developed and are currently under investigation in clinical trials. Farnesyl transferase inhibitors (FTIs) are the best studied drugs and two orally bioavailable FTIs (tipifarnib and lonafarnib) are being tested in the combination with cytotoxic drugs in phase 3 clinical trials in lung cancer.

BRAF protein serine/threonine kinase is a downstream effector of the *RAS* pathway. Mutations of *BRAF* occur in ≈70% of melanoma cases, and in some other cancers, including 2%–3% of NSCLCs (mostly adenocarcinomas). An orally administered RAF kinase inhibitor, sorafenib, is being tested in phase 1 and 2 trials in lung cancer.

Activated BRAF phosphorylates and activates MEK1 and MEK2, which in turn phosphorylate and activate ERK1 and ERK2. Activated ERK1/ERK2 phosphorylate various downstream substrates involved in mitogenic signal transduction and cell survival. ERK1 and ERK2 are constitutively activated in a subset of lung cancer cell lines and thus MEK and ERK are therapeutic targets for lung cancer treatment. An oral MEK inhibitor, CI-1040, and its derivative, PD03255901, are being tested in clinical trials for lung cancer.

The PI3K/Akt/PTEN/mTOR Pathway

Phosphatidylinositol-3-kinases (PI3Ks) are lipid kinases that regulate several cellular processes such as proliferation, growth, apoptosis and cytoskeletal rearrangement. *PIK3CA*, which encodes the alpha catalytic subunit of PI3K, is mutated in 3%–4% of ≈260 NSCLC samples (16). Mutation in *PIK3CA* results in elevated

lipid kinase levels, and introduction of mutant *PIK3CA* transforms NIH-3T3 cells, demonstrating its oncogenic ability. Thus, mutant *PIK3CA* is an attractive therapeutic target in tumor cells with such mutations.

Akt/protein kinase B (PKB) is a downstream effector of PI3Ks and its activation has oncogenic effects. Constitutive activation of Akt was reported in 16 of 17 NSCLC cell lines (17). Active Akt promotes cellular survival and a PI3K inhibitor, LY294002, which reduces Akt phosphorylation, enhances the sensitivity of NSCLCs to chemotherapeutic agents and radiation therapy. Thus, PI3K inhibitors may be useful as cytotoxic and/or chemosensitizing agents for NSCLCs (17). By contrast, the tumor suppressor gene (TS6) *PTEN* is a negative regulator of Akt; thus, loss of *PTEN* activity provides another way of activating the Akt pathway. Although *PTEN* is only infrequently mutated in SCLC and NSCLC, greatly reduced or loss of *PTEN* protein expression by unknown mechanisms is common in lung cancers. Thus, lung tumors with loss of *PTEN* activity are also candidates for therapy targeting this pathway.

Mammalian target of rapamycin (mTOR) is a key downstream target of Akt kinase activity and a central regulator of cell growth. mTOR is another potential therapeutic target in the PI3K pathway. mTOR inhibitors, such as rapamycin (sirolimus) and its derivatives (CCI-779, RAD001, and AP23576), have pre-clinical antitumor activity in lung cancer, and these drugs are being evaluated in clinical trials for lung cancer treatment.

Signal Transducers and Activators of Transcription Family

The signal transducers and activators of transcription (STAT) family consists of seven members: STAT1, 2, 3, 4, 5A, 5B, and 6. STATs are cytoplasmic proteins that are activated by tyrosine phosphorylation, resulting in dimer formation and translocation to the nucleus to regulate the expression of target genes. Constitutive activation of STAT3 and STAT5 contributes to oncogenesis in a wide range of malignancies including lymphoma, breast, and lung cancers, by stimulating cell proliferation and inhibiting apoptosis through up-regulation of genes involved in these pathways, such as *BCL XL*, *Cyclin D1*, and *MYC*. Constitutive DNA-binding activity of STAT3 was shown in six of seven NSCLC cell lines, and inhibition of STAT3 with antisense oligonucleotides against STAT3 or a dominant-negative STAT3 resulted in apoptosis in NSCLC cell lines with constitutively activated STAT3 (18). In addition, it has been shown that STAT3 activation is required for cell survival and growth of NSCLC cell lines with mutant *EGFRs* (19). Thus, activated STAT3 is another therapeutic target, perhaps in combination with EGFR-targeted therapy.

MYC Family

The *MYC* gene family encodes three nuclear phosphoproteins (*MYC*, *MYCN*, and *MYCL*), which heterodimerize with MAX proteins to function as transcription factors. They play a role in cell proliferation, apoptosis and development of human tumors. Amplification of one member of *MYC* family occurs in 18% to

31% of SCLCs and 8% to 20% of NSCLCs (5). *MYC* amplification occurs in SCLC and NSCLC whereas *MYCN* and *MYCL* amplifications nearly always occur only in SCLC. *MYC*-targeted drugs need to be developed and tested in lung cancer.

Abnormalities in Growth-Inhibitory Tumor-Suppressor Pathways (Inactivation of Tumor-Suppressor Genes)

Several key tumor-suppressor pathways are frequently inactivated in lung cancer. These include the *p53* and the *p16^{INK4a}*-cyclin D1-CDK4-RB pathways.

The *p53* Pathway

The TSG *p53*, located on chromosome 17p13.1, encodes a protein that functions as a transcription factor, and *p53* expression is induced in response to DNA damage. The induction of *p53* results in the expression of downstream genes (e.g., *p21/WAF/CIP1*, *GADD45*, *BAX*), leading to a cell cycle arrest to permit repair or apoptosis. Thus, loss of *p53* function allows the genetically damaged cells to grow, leading to the clonal expansion of premalignant or malignant cells. *p53* is altered in $\approx 90\%$ of SCLCs and $\approx 50\%$ of NSCLCs (5,20). Most of these alterations are missense point mutations (70%–80%), most of which affect DNA binding domain (exons 5–8), and occasionally homozygous deletions. *p53* mutations in lung cancer correlate with cigarette smoking, and G-T transversions expected from tobacco smoke carcinogens are frequently found in lung cancers of smokers compared with lung cancers from nonsmokers (5,20). In most lung cancers only mutant *p53* allele is present because of loss of the wild-type *p53* allele. However, mutant *p53* proteins can form heterodimers with wild-type *p53* and thus inactivate wild-type function.

When wild-type *p53* is re-expressed in lung cancer cells with mutant or deleted *p53*, the tumor cells undergo apoptosis. These findings led to clinical trials of *p53* replacement therapy, which showed evidence of antitumor activity and the feasibility and safety of *p53* gene therapy. INGN 201 (Ad5CMV-*p53*, Advexin), a replication-impaired *p53* adenoviral vector has been evaluated in clinical trials and is safe and effective for the treatment of several different types of cancer, including lung cancer (21). However, clear clinical benefit of this treatment in randomized controlled studies has yet to be shown.

Two important upstream regulators in the *p53* pathway are MDM2 and *p14^{ARF}*. MDM2 functions as an oncogene by reducing *p53* levels through enhanced proteasome-dependent degradation. Amplification of *MDM2* was reported in $\approx 7\%$ of NSCLCs, resulting in loss of *p53* function (20). TSG *p14^{ARF}* is an alternative reading frame gene of the *p16^{INK4a}* locus (9p21) and encodes a protein that enhances *p53* activity by binding to MDM2, leading to the stabilization of *p53*. Immunohistochemistry analyses of *p14^{ARF}* on lung cancers have shown that *p14^{ARF}* protein expression

was lost in $\approx 65\%$ of SCLCs and $\approx 40\%$ of NSCLCs. Thus, loss of *p14^{ARF}* would also lead to loss of *p53* expression and function.

The *p16^{INK4a}*-Cyclin D1-CDK4-RB Pathway

The *p16^{INK4a}*-cyclin D1-CDK4-RB pathway consists of RB and three of its associated upstream products and plays a central role in G₁/S cell transition. The RB gene was initially identified as a TSG in retinoblastoma, and one of the four components of this pathway is frequently altered in nearly all lung cancers. Hypophosphorylated RB exerts its tumor-suppressor ability by binding and controlling E2F transcription factor, which is essential for G₁/S transition. Once RB is hyperphosphorylated by Cyclin D1/CDK4 complex, it releases E2F, resulting in transition from G₁ to S. Absent or mutant RB protein is found in $\approx 90\%$ of SCLCs and approximately 15% to 30% of NSCLCs, leading to loss of the G₁/S checkpoint (5). Another regulator of RB function is *p16^{INK4a}*, which keeps RB in the unphosphorylated (and growth-suppressing) mode by preventing CDK4 from phosphorylating RB. Inactivation of *p16^{INK4a}* is caused by biallelic deletion, intragenic mutations, and promoter hypermethylation, resulting in loss of the RB pathway. In contrast to RB, *p16^{INK4a}* is very frequently inactivated in NSCLCs ($\approx 70\%$) but rarely altered in SCLCs (5). Overexpression of CDK4 or Cyclin D1 inhibits the RB pathway by blocking the growth-suppressing activity of *p16^{INK4a}*. High-level amplification on CDK4 has been reported in $\approx 2\%$ of NSCLCs (22). Immunohistochemistry analysis shows that Cyclin D1 is overexpressed in more than 40% of NSCLCs (5). Overexpression of Cyclin D1 in normal-appearing bronchial epithelium of patients with NSCLC was associated with smoking, suggesting the possible utility of Cyclin D1 as a molecular marker to identify individuals at high risk of developing lung cancer (23).

The 3p Tumor Suppressor Genes

Allele loss in 3p, including loss of heterozygosity (LOH) or homozygous deletion, occurs in nearly 100% of SCLCs and more than 90% of NSCLCs and is one of the earliest events for lung cancer development. Three discrete regions of 3p loss have been identified by allelotyping in lung cancers, including a 600-kb segment in 3p21.3, the 3p14.2 (*FHIT/FRAB3*), and 3p12 (*ROBO1/DUTT1*) regions. The 3p21.3 region has been analyzed extensively, and 25 genes were identified from this region, including TSGs *RASSF1A*, *FUS1*, *NPRL2*, *101F6*, *SEMA3B*, and *SEMA3F*. Because of the early change in 3p chromosome regions (occurring in histologically normal lung epithelium) the presence of 3p allele loss and inactivation of expression of these 3p TSGs can be of use in identifying a bronchial epithelial field defect caused by smoking. One of the best studied of these genes is *RASSF1A*, which is involved in cell cycle, apoptosis, and microtubule stability, and is rarely mutated in lung cancer but whose expression is frequently lost by tumor-acquired promoter methylation in $\approx 90\%$ of SCLCs and $\approx 50\%$ of NSCLCs (24). Re-expression of *RASSF1A* suppresses the growth of lung cancer cell lines in vitro and in vivo (24). *FUS1* is directly next to *RASSF1A* and one of the two alleles of the gene is often lost in lung cancers. Although *FUS1*

is only rarely mutated in lung cancers and its mRNA is usually expressed, its protein expression is frequently lost in lung cancer. Wild-type *FUS1* but not tumor-acquired mutant *FUS1* induces G₁ growth arrest and apoptosis. Administration of *FUS1* with the nonviral vector, DOTAP: cholesterol (DOTAP: Chol) nanoparticle (DOTAP:Chol-*FUS1*) inhibits cancer cell growth in vitro and in vivo, providing the rationale for using *FUS1* gene therapy for systemic treatment for lung tumors with *FUS1* nanoparticles being tested in clinical trials (25). *SEMA3B* and *SEMA3F* are also located at 3p21.3 (close to *RASSF1A* and *FUS1*), and they are extracellular secreted members of the semaphorin family, important in axonal guidance. Wild-type *SEMA3B* but not tumor-acquired single-amino-acid missense mutants of *SEMA3B* induces apoptosis when re-expressed in lung cancers or added as soluble molecules (26). Likewise, *SEMA3F* inhibits the growth of lung cancer cells in xenografts (27). One mechanism of *SEMA3B* tumor inhibition is through its ability to block vascular endothelial growth factor (VEGF) autocrine activity (26). Since *SEMA3B* and *SEMA3F* are soluble and secreted proteins, they are promising candidates as drugs for systemic treatment. Other 3p genes with good evidence for tumor-suppressor activities are *FHIT* and retinoic acid receptor β (*RAR\beta*). Expression of *FHIT* protein is lost in $\sim$ 50% of lung cancers because of homozygous deletion, promoter methylation, or aberrant transcripts. Re-expressed *FHIT* induces apoptosis in lung cancer. The *RAR\beta* gene is located in 3p24 region and functions as a nuclear receptor for retinoic acid (RA). Although the *RAR\beta* gene is not mutated in lung cancer, it undergoes promoter methylation in 72% of SCLCs and 41% of NSCLCs, leading to loss of its expression. Re-introduction of *RAR\beta2* into lung cancer cells suppresses their growth in vitro and in vivo.

Other Tumor Suppressor Genes (*STK11/LKB1*, *TSLC1*, and *SLIT2*)

The serine-threonine kinase *STK11/LKB1*, responsible for Peutz-Jeghers syndrome, is frequently (33%) mutated in lung adenocarcinomas but not in other NSCLC histologies (28). Re-expression of *STK11/LKB1* suppresses cyclooxygenase-2 induction and cellular invasion but not proliferation of lung cancer cells. *TSLC1*, encoding a member of the immunoglobulin superfamily molecules, is rarely (1.2%) mutated in lung cancers, but its expression is frequently lost or reduced by LOH and/or promoter hypermethylation (29). *SLIT2* encodes a secreted protein, which plays an important role in axon guidance (30). *SLIT2* is not mutated but is frequently methylated in NSCLC, and overexpression of *SLIT2* suppresses the growth of NSCLC cell lines that lack *SLIT2* expression (30). Since *SLIT2* is a secreted protein, it is a potential therapeutic for lung cancer.

Epigenetic Changes in Lung Cancer: DNA Promoter Methylation Change as a Mechanism for Inactivating Tumor-Suppressor Genes

Tumor-acquired DNA methylation near the transcription start site of genes (aberrant hypermethylation) that suppresses gene expression is probably the most common molecular event in human cancers. Most lung cancers have multiple aberrantly methylated

genes. Over 80 genes are reported to be hypermethylated in lung cancer compared with normal lung, including well-studied genes such as *RAR\beta*, *TIMP3*, *p16^{INK4a}*, *RASSF1A*, *MGMT*, *FHIT*, *DAPK*, *ECAD*, and *GSTP1*. Detecting methylated DNA sequence in biologic fluids (sputum or blood) is potentially a powerful tool for early detection of lung cancer. Hypermethylation of *p16^{INK4a}* can be detected in sputum or exfoliated lung cells prior to lung cancer diagnosis. Detection of methylation of three or more genes in the sputum of smokers increased the risk of developing lung cancer by 6.5-fold with a sensitivity and specificity of 64% (31). This promising result warrants further prospective studies using an optimized set of genes. Detection of aberrant hypermethylation of TSGs in serum DNA, such as aberrantly methylated *p16^{INK4a}*, is also a promising tool for early detection of lung cancer and thus needs to undergo controlled studies. Using genome-wide approaches we have identified 132 genes, the majority of which are methylated in lung cancer compared with normal lung with high specificity and thus give a large new diagnostic panel of genes to use for early detection (32).

In contrast to gene mutation, promoter hypermethylation is a reversible phenomenon, making this an attractive target for cancer therapy. In fact, an inhibitor of DNA methylation, azacitidine (Vidaza) is shown to prolong the survival of patients with myelodysplastic syndrome. However, its efficacy in lung cancer has yet to be demonstrated. Treatment with decitabine, another inhibitor of DNA methylation, given every 3 weeks in combination with cisplatin for solid tumors including NSCLC did not show significant antitumor activity (33). However, continuous treatment rather than an intermittent schedule needs to be tested. Finally, other epigenetic mechanisms, such as histone deacetylation, also play roles in gene inactivation. Thus, clinical trials with histone deacetylase inhibitors, such as depsipeptide, are under way in lung cancer.

Evading Apoptosis

In addition to uncontrolled growth, evading apoptosis is another important oncogenic property of cancer cells. *p53* and *BCL2* are the two important genes involved in apoptosis pathways. *BCL2* protein inhibits apoptosis and is overexpressed in SCLC (75%–95%) and NSCLC (10%–35%; 5,20). Antisense-oligonucleotide against *BCL2*, oblimersen sodium (G3139; Genasense), enhances the efficacy of standard chemotherapy in several cancers, including lung cancer (34). Randomized phase 2 trials of oblimersen in combination with cytotoxic chemotherapy for SCLC and NSCLC are being conducted. ABT-737, a potent inhibitor of Bcl-2, Bcl-X_L, and Bcl-w, has shown efficacy in xenograft models of SCLC and enhances the activity of paclitaxel against A549 NSCLC cells, providing a rationale for its use in clinical trials for lung cancer as monotherapy or in combination with cytotoxic drugs (35).

Activation of Telomerase

Immortalization (limitless replicative potential) is one of the “hallmarks of cancer” and is believed to be one of the initial steps of transformation (36). The most critical component regulating

limitless replication capability is telomere maintenance regulated by telomerase (37). The enzyme telomerase maintains telomeric repeats by elongating telomeric DNA by reverse transcription, and its activity is largely determined by the expression levels of hTERT, which is the protein catalytic subunit of telomerase (37). Approximately 80% of NSCLCs and nearly 100% of SCLCs have detectable levels of telomerase whereas most normal human cells do not express sufficient telomerase activity to maintain telomeres. In addition, high levels of telomerase activity in primary NSCLCs are observed in advanced pathologic stages, suggesting that expression of telomerase may contribute to the later stages of lung cancer progression (38). Thus, telomerase is an important therapeutic target. The novel telomerase template antagonist, GRN163L, which targets RNA template region of hTR, inhibits anchorage-independent growth and *in vivo* xenograft tumor growth of lung cancer cells (39), providing the rationale for its use in clinical trials of lung cancer treatment. This treatment also should be effective against lung cancer stem cells (see following sections).

Sustained Angiogenesis

One of the “hallmarks of cancer” is the ability to stimulate angiogenesis (36). Elevated angiogenesis in lung cancer measured by microvessel density correlates with the incidence of metastasis and poor survival. VEGF is the most potent pro-angiogenic factor and is frequently produced at high levels by lung cancers. Bevacizumab, a monoclonal antibody that neutralizes all VEGF isoforms, has been tested clinically for lung cancer treatment. The addition of bevacizumab to chemotherapy with paclitaxel and carboplatin in patients with nonsquamous cell advanced NSCLC provided a statistically and clinically significant survival advantage (40). The results of this study were dramatic and provided for approval of bevacizumab as a lung cancer therapeutic as well as being a major impetus to continue work on VEGF antagonists in lung cancer. Also dramatic were the occasional patients who had life-threatening pulmonary hemorrhage from bevacizumab treatment, indicating that this therapy must be carefully chosen and monitored. Another therapeutic approach for angiogenesis is to inhibit VEGF receptor (VEGFR) activation by TKIs. ZD6474 is a dual-kinase inhibitor that targets VEGFR and EGFR. The combination of ZD6474 and docetaxel as a second-line therapy in a phase 2 lung cancer clinical trial improved progression-free survival in patients with advanced disease.

New Techniques and Models for Studying Lung Cancer Pathogenesis

Genome-Wide Approaches for Identifying Regions of Genetic Changes

Karyotypic studies provided the first information about genetic changes and genetic instability in lung cancer, revealing multiple cytogenetic changes in most SCLCs and NSCLCs. In SCLCs, losses from 3p, 5q, 13q, and 17p occur frequently together with

double minutes associated with amplification of the *MYC* family genes. In NSCLCs, deletions of 3p, 9p, and 17p, +7, i(5)(p10), and i(8)(q10) occur frequently. Cytogenetic techniques using comparative genomic hybridization (CGH) allowed analyzing gains and losses of chromosomes precisely as well as quantification of these changes, revealing gains in 5p, 1q24, and Xq26, and deletions in 22q12.1–13.1, 10q26, and 16p11.2 in SCLCs. Moreover, as reviewed by Thomas et al., recent advances in array-based high-throughput techniques including array-based CGH and SNP array analysis have identified even smaller regions with genomic alterations (41). Efforts have focused on identifying the specific genes with abnormalities in these regions.

Gene Expression Profiling by Microarray Technology

Oligonucleotide and cDNA microarrays are widely available and powerful tools for analyzing global gene expression changes in cancers compared with normal tissues. Possible applications of this technology in cancer research include (1) classifying subtypes of cancer; (2) predicting prognosis; (3) predicting the response of cancers to therapeutic interventions; (4) finding markers for early detection of cancer; and (5) finding new TSGs or oncogenes involved in cancer pathogenesis. In addition, combining array expression data and genome-wide genetic alteration data obtained by array-based CGH and SNP analysis will allow identification of key molecular changes.

As reviewed by Meyerson (42), three studies using microarray technology demonstrated that gene expression patterns recapitulate conventional morphologic classification of lung tumors into squamous, large cell, small cell, and adenocarcinoma. Two of these studies found that adenocarcinomas could be placed in subgroups that correlated with patient survival (42). From the clinical perspective, it is vital to identify patients with lung cancer at early stage who are at high risk of relapse because such patients are more likely to benefit from adjuvant chemotherapy. To predict the prognosis of early-stage NSCLC patients Potti et al. developed a new “metagene” model that integrates multiple gene-expression profiles with various clinical variables (43). They showed that the model predicted recurrence significantly better than a model that included only clinical data. This promising result warrants testing of this model in larger patients series.

Several studies have identified sets of genes in lung cancer whose expression levels are associated with response to antitumor drugs. Furthermore, gene expression signatures involved in oncogenic pathways such as RAS and MYC pathways were described and were found to be significantly correlated with the prognosis of patients and sensitivity to cytotoxic drugs in three types of cancers, including NSCLC (44). These results provide a basis for using tumor gene expression signatures as a guide to select therapeutics for patients with these tumors.

Spira et al. compared the gene expression profiles of human airway epithelial cells obtained by bronchoscopy from current, former, and never-smokers and identified approximately 100 genes differentially expressed between current and never smokers (45). They also found that changes in expression levels of several smoking-induced genes, including potential TSGs and oncogenes,

persisted after cessation of smoking, which could be a possible explanation for the fact that approximately 50% of all new cases occur in former smokers. Recently, the SIEGE (Smoking Induced Epithelial Gene Expression) database was created. The SIEGE database has Affymetrix array data on bronchial epitheliums from current, former, and never-smokers and their relevant clinical data. The long-term goal of this database is to provide an airway gene expression signature that predicts the risk of lung cancer development among smokers.

New Transgenic Mouse Models of Lung Cancer

Mouse models that recapitulate the carcinogenic process of human lung cancer are powerful tools to improve our understanding of lung cancer pathogenesis, develop targeted therapeutics, and evaluate their *in vivo* efficacies. Several different types of transgenic mouse models for studying lung cancer have been developed with innovative strategies. Bitransgenic models using Cre/LoxP recombination or tetracycline-inducible gene expression system have enabled regulating the expression of a gene in mice in a timely and spatially controlled manner. Two groups engineered mouse strains harboring conditional mutant *K-ras* alleles that are expressed only after Cre/LoxP-mediated recombination occurs. Both groups showed that oncogenic *K-ras* activation induces lung adenocarcinoma, demonstrating the contributions of oncogenic *K-ras* to lung cancer pathogenesis (46). Moreover, Meuwissen et al. developed a mouse model of SCLC by inactivating both *Rb* and *p53* using Cre/LoxP recombination system (46). Using a tetracycline-inducible gene expression system, mice harboring *EGFR* tyrosine kinase domain mutations were engineered. These mice developed adenocarcinomas very similar to human adenocarcinomas with *EGFR* mutations. Although there can be significant differences in lung tumor development between humans and mice, mouse models have a complete physiologic environment and allow analyzing host

tumor interaction and angiogenesis, which cannot be studied in tissue culture. Finally, no mouse model of squamous cell carcinoma of the lung has been developed.

Immortalized Human Bronchial Epithelial Cell Models for Studying Lung Cancer Pathogenesis

To systemically test the importance of the multiple gene alterations found in lung cancer, we developed a series of human bronchial epithelial cell (HBEC) lines immortalized with *cdk4* (providing a bypass of *p16^{INK4A}*) and *hTERT* (Figure 30-2A). Both *p16^{INK4A}* loss-of-function and telomerase expression are almost universally altered in human lung cancers (47). These HBEC lines are immortal, can be cloned and genetically manipulated but do not form soft-agar colonies or tumors in nude mice, and can differentiate into a pseudostratified epithelium structure, very similar to normal human bronchial epithelium in organotypic three-dimensional (3D) culture (Figures 30-2B and 30-2D; 11,48). One of the great advantages of this system is that immortalization is performed without using viral oncoproteins. However, since Taiwanese women with lung adenocarcinoma were found to have oncogenic HPV in their lung cancers, we have also prepared immortalized HBEC lines with HPV (47). This is an attractive model system for analyzing the multistep pathogenesis of lung cancer. For example, HBEC lines manipulated to have mutant *KRAS^{V12}*, *p53* knockdown, or mutant *EGFR* alone or in various combinations acquired the ability to achieve higher cell density in culture, grow in soft agar, and invade in 3D organotypic cultures (Figures 30-2C and 30-2D; 11). However, the combination of four alterations (*p16* bypass, telomerase, *p53* abrogation, and mutant *KRAS* or mutant *EGFR*) was not sufficient to cause the cells to form tumors in nude mice (11). These results indicate that the HBEC system is a powerful new approach to assess the contribution of individual and combinations of alterations to lung cancer pathogenesis. Another possible utility of the HBEC system is to evaluate the ability

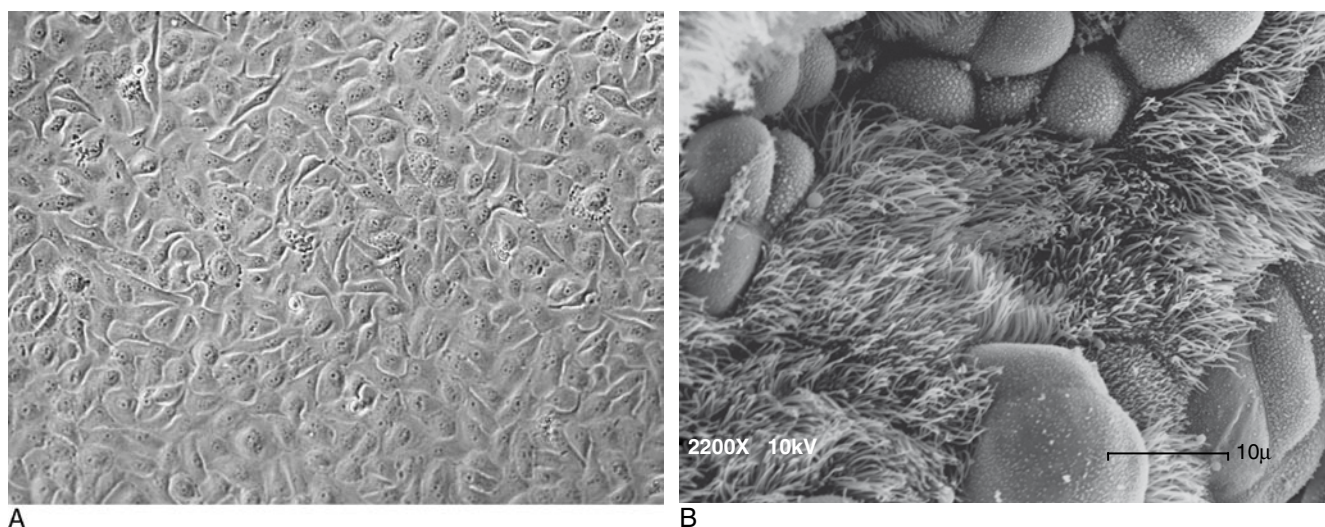


FIGURE 30-2 A: Photomicrograph of human bronchial epithelial cells (HBECs) immortalized with *Cdk4* and *hTERT*. These cells have an epithelial morphology and form a monolayer in confluent culture. (From Ramirez RD et al. *Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins*. *Cancer Res* 2004;64:9027, with permission.) **B:** Scanning electron microscopy of the apical surface of HBECs in organotypic three-dimensional (3D) culture showing ciliated cells (middle) and goblet (mucus-producing) cells (upper and lower). (From Vaughan MB, et al. *A three-dimensional model of differentiation of immortalized human bronchial epithelial cells*. *Differentiation* 2006;74:141, with permission.)

(Continued)

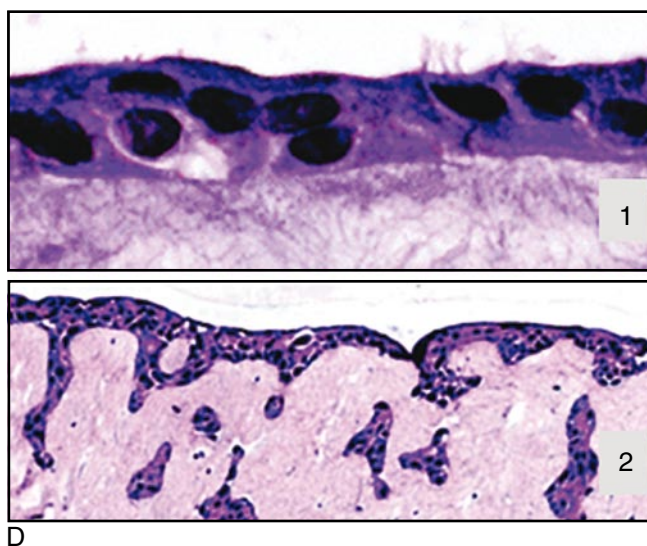
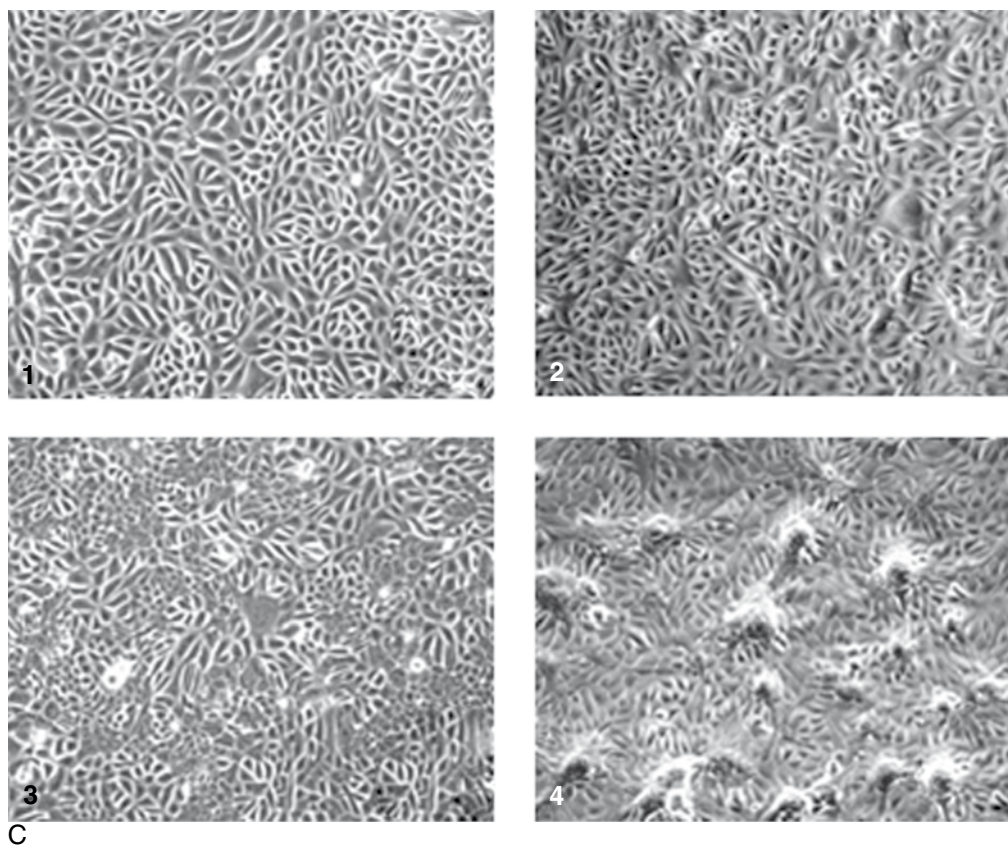


FIGURE 30-2—CONT'D C: Photomicrographs of HBECs expressing: 1, vector; 2, p53 RNAi; 3, mutant K-RAS^{V12}; and 4, p53 RNAi and mutant K-RAS^{V12}. The combination of p53 RNAi and mutant K-RAS^{V12} significantly increases saturation density of the HBECs. (From Satom, et al. *Multiple oncogenic changes (K-RAS (V12), p53 knockdown, mutant EGFRs, p16 bypass, telomerase) are not sufficient to confer a full malignant phenotype on human bronchial epithelial cells. Cancer Res 2006;66:2116, with permission.*) **D:** Effect of p53 knockdown and mutant K-RAS^{V12} on 3D organotypic culture of HBEC3 cells. 1, stained paraffin cross-sections of organotypic cultures of HBEC3 cells show that they formed a confluent layer of cells on the upper surface of the culture with the presence of cilia-like structures. 2, p53 RNAi and mutant K-RAS^{V12}-expressing HBEC3 cells show a histologic change similar to metaplasia and dysplasia as they invade into the fibroblast and collagen underlayer. (From Satom, et al. *Multiple oncogenic changes (K-RAS (V12), p53 knockdown, mutant EGFRs, p16 bypass, telomerase) are not sufficient to confer a full malignant phenotype on human bronchial epithelial cells. Cancer Res 2006;66:2116, with permission.*)

of carcinogens to transform normal epithelial cells to malignant or premalignant cells. Since HBEC cells do not form soft-agar colonies but become able to do so after oncogenic manipulations, one would be able to evaluate the ability of carcinogens to transform normal HBECs by performing soft-agar analysis on these cells.

Cancer Stem Cell Model and Lung Cancer

Evidence for cancer stem cells was first demonstrated in hematologic malignancies. Subsequently, cancer stem-like tumor-initiating cells have been identified in breast and central nervous system tumors. This evidence supports the hypothesis that only a subset

of malignant cells within a tumor has the ability to undergo self-renewal and form metastasis whereas most cells in a tumor are short-lived and do not have such abilities. Although direct evidence for the existence of cancer stem cells in lung cancer has yet to be shown, Kim et al. have isolated a stem cell population in the *Kras*^{V12} transgenic mouse model of lung cancer, at the region of bronchioalveolar duct junction. This population has the ability to undergo self-renewal and differentiation and are referred as to BASCs (bronchioalveolar stem cells; 49). Furthermore, they have shown that introduction of oncogenic *Ras* caused these putative stem cells to expand, suggesting that these cells are the precursor of adenocarcinoma of the lung. The concept of cancer stem cell has important implications in cancer treatment. The cancer stem cell model hypothesizes that cancer stem cells can escape from the effects of chemotherapy and radiotherapy because of their low proliferation rate and potential drug resistance related to drug transporter expression and thus can regenerate tumors. If it is the case, therapeutic interventions that target cancer stem cells are needed. To develop such treatment methods, methodologies for isolating and characterizing cancer stem cells in lung cancer are being developed.

Conclusions and Future Perspectives

Extensive molecular analysis of lung cancer has provided a great deal of information on molecular abnormalities occurring in lung cancer, offering a rationale for developing new methods of diagnosis, prevention, and treatment. Attempts to translate such knowledge into the clinical setting are being made. Examples include analyzing the methylation status of a set of genes in sputum samples, which were shown to improve early detection of lung cancer, survival benefit of patients with nonsquamous advanced NSCLCs

treated with a monoclonal VEGF inhibitor, bevacizumab, and EGFR TKIs, which give dramatic clinical responses in patients whose tumors have *EGFR* TK domain mutations. To more efficiently translate the molecular findings in lung cancer into clinical applications, several important issues need to be addressed. First, developing efficient methods to identify patient subpopulations that are most likely to benefit from targeted drugs is crucial. Global strategies such as microarray and proteomics analyses on tumor or blood specimens from individual clinical sample may prove useful. In addition, exploring efficient combinations of drugs or targeted therapies is necessary. Since lung cancers have undergone multiple genetic changes, it is reasonable to combine more than one targeted therapy. Most important, we need to perform global searches for targets that are absolutely required for lung cancer malignant behaviors. The HBEC model system may be useful in identifying such targets because of the ability to identify changes that have the most impact on tumorigenicity of bronchial epithelial cells. Finally, the molecular factors that are specific for a cancer stem cell population in lung cancer could be the ultimate targets for therapy because cancer stem cells are believed to have the ability to initiate and maintain cancers, and thus eradicating these cells should enable us to cure patients.

Acknowledgment

This chapter was developed and uses details from Sato et al. A translational overview of molecular pathogenesis of lung cancer, *J Thoracic Oncol* 2007;2:327–343(50). This work was supported by Lung Cancer SPORE P50CA70907, DOD VITAL (W81XWH041014202PP), PROSPECT, NASA NSCOR (NNJ05HD36G), DOE, and Gillson Longenbaugh Foundation.

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Molecular Abnormalities in Colon and Rectal Cancer

Colorectal cancer development is a complex process. Causal agents and mechanisms include environmental and dietary factors, and inherited and somatic mutations. Great progress has been made in the last 25 years toward defining the constellation of molecular alterations contributing to colorectal tumor development. Specific oncogene and tumor-suppressor gene mutations have been identified in tumors of various stages. Activation of cellular proto-oncogenes can result from specific point mutations and rearrangements that alter gene structure and function or from chromosomal rearrangements and gene amplifications that disrupt the regulated expression of the proto-oncogene. Tumor-suppressor gene inactivation can result from localized mutations or complete loss of the gene. At present, only somatic (arising in non-germ cells during the patient's lifetime) mutations in proto-oncogenes have been detected in colorectal tumors. Although most tumor-suppressor gene mutations in colorectal cancer are also somatic, inherited mutations in tumor-suppressor genes have a critical role in colorectal cancer predisposition syndromes.

Principle objectives of this chapter are to review the following: (1) the epidemiology of colorectal cancer; (2) the sequence of histopathologic alterations in the course of the progression to malignancy; (3) the molecular alterations present in colorectal tumors of various stages and their relevance to selected hereditary cancer syndromes; and (4) the potential clinical utility of the genetic alterations in early detection and clinical management of patients and families affected by colorectal cancer.

Epidemiology

Colorectal cancer is the second leading cause of cancer deaths in the United States (1). In 2006, it is anticipated that nearly 150,000 individuals in the United States will be diagnosed with colon or rectal cancer, and about 55,000 will die from the disease (1). Male and female incidence and survival are almost identical (1). Important differences in prognosis have been observed in different racial groups in the United States (1). These differences persist even after accounting for the stage of disease at diagnosis and are likely to reflect a combination of factors, including access to care, differences in treatment, and perhaps molecular and biologic features of the cancers (2).

Most cases of colorectal cancer are considered to be sporadic, indicating that clear-cut familial or genetic predisposition factors are not readily apparent. Between 15% and 30% of cases are considered to have a major hereditary component, based on the occurrence of similar cancers in first- or second-degree relatives (3). Less than a fourth of these familial cases (i.e., less than 5% of all cases) occur in a setting with a family history and/or clinical features indicative of a highly penetrant, single-gene-mutation cancer syndrome predisposing to colorectal carcinoma development. Gene alterations or DNA sequence variations with key contributing roles in most familial cases remain to be defined. It appears that the familial cases may be a heterogeneous group, in which modest to moderate predisposition to colorectal carcinoma is possibly conferred by an undetermined number of potentially common genetic variations.

In addition to family history, other risk factors for colorectal cancer include older age, chronic inflammatory bowel disease, and a diet rich in unsaturated fats and red meat. Evidence for a protective effect has been presented for nonsteroidal anti-inflammatory drugs (NSAIDs) in many studies and HMG-CoA reductase inhibitors in a few studies (4). The unfortunate reality is that the principle dietary and environmental factors contributing to colorectal cancers in the United States and other Western countries are rather poorly defined, and upwards of 75% of all incident colorectal cancers arise in people with no well-defined risk factor. On a more optimistic note, a large fraction of colorectal cancer deaths seems to be preventable by early detection. By some estimates, improved adoption and implementation of current screening recommendations for colorectal cancer could save thousands of lives per year (5).

Histopathologic Changes in Colorectal Carcinogenesis: the Adenoma-Carcinoma Sequence

The surface of the large intestine is covered by an epithelium, characterized by finger-like invaginations into the underlying stroma; the invaginations are termed "crypts." Stem cells near the base of each crypt give rise to distinct cell types required for the fulfillment of the intestine's resorptive, secretory, and endocrine functions.

During their differentiation, epithelial cells migrate from the base of the crypt to the surface, and the cells are ultimately shed into the gut lumen. With the exception of the one to five stem cells per crypt, the entire epithelial lining is turned over within a few days.

Among the different types of benign gastrointestinal lesions, a generic term for any localized lesion protruding above the surrounding mucosal surface is “polyp.” Most polyps, particularly small polyps 5 mm or smaller, are of the hyperplastic type, with characteristic serrated glands and distended goblet (mucus-producing) cells. However, it is the adenomatous polyp or adenoma that is believed to be the major precursor lesion to carcinoma. Both gross and histopathologic features can be used to distinguish adenomas. Grossly, the size is measured and its morphology can be described as pedunculated (with a stalk), sessile (flat), or semisessile (6). Among the histopathologic features, the degree of dysplasia and glandular architecture are used to distinguish tumors and may be useful for predicting the likelihood that a lesion contains a focus of cancer or its risk of progression to cancer.

The proposal that most cases of colorectal cancer arise from adenomatous lesions is supported by at least three independent lines of evidence. First, a few longitudinal studies have assessed the risk of subsequent colorectal cancer development in individuals with adenomatous polyps (7). In these studies it became apparent that patients who did not have their adenomas removed, had an approximately eightfold increased risk of colorectal cancer compared with the group that had their adenoma removed. Notably, after polypectomy, patients did not show an increase in colorectal carcinoma incidence in comparison with a control group without adenomas, suggesting that removal of adenomatous polyps had a therapeutic effect (7). Second, histopathologic studies have shown that foci of carcinoma can often be detected in adenomatous polyps, particularly those with increased size, dysplasia, and villous histopathology. Third, individuals affected by syndromes that strongly predispose to the development of hundreds of adenomas, such as familial adenomatous polyposis (to be discussed in subsequent sections), invariably develop colorectal cancers by the third to fifth decades of life, if their colons are not removed.

Some studies have suggested that patients who develop hyperplastic polyps may have an increased risk of adenomas (8,9). Nevertheless, only adenomatous polyps have a clearly increased risk of progressing to cancer (10). However, patients with numerous hyperplastic polyps (e.g., patients with juvenile hyperplastic polyposis syndrome) have a clearly increased colorectal carcinoma risk. It has been increasingly appreciated that a subtype of polyps, termed “sessile serrated adenomas,” which share some morphologic features with hyperplastic polyps, does indeed show an increased cancer risk (12). Most carcinomas arising from these so-called serrated adenomas seem to be associated with distinct molecular defects that will be discussed later in the chapter (CpG island methylation phenotype, CIMP; 13,14).

At least two other pathways leading to colorectal cancer and that are not associated with overt development of adenomatous polyps as precursors are evident: chronic inflammatory bowel diseases (particularly ulcerative colitis [UC] and to a lesser extent Crohn disease) and flat adenoma syndromes. Ulcerative colitis is a chronic

inflammatory disease of largely unknown etiology. The possible precursor lesions to cancer in patients with UC include dysplasia and flat adenomatous plaques (6,15). In a subset of patients with hereditary cancer syndromes, as well as some sporadic cases, colon cancer seems to develop directly from flat adenoma or intraepithelial dysplasia. In these cases the progression from benign lesion to overt carcinoma seems to be significantly faster than in the normal adenoma-carcinoma progression (6).

Hereditary Colorectal Cancer Syndromes and Molecular Pathways of Colorectal Carcinogenesis

It is now generally accepted that somatic mutations drive the initiation and progression of tumor development. The repertoire of gene defects that commonly occur in colorectal carcinogenesis has been largely defined (16). However, only a limited subset of this collection of gene defects is usually present in a single tumor. The genetic alterations of greatest interest are those that are clonal in tumors (i.e., present in all, or nearly all, neoplastic cells of a primary tumor at a given stage, but not present in the normal cells of the patient). It is inferred that such clonal genetic alterations are causal in promoting further tumor outgrowth/progression, because somatic mutations can only become clonal by a limited number of mechanisms. The genetic alteration itself could have been selected for because it provided the cell with a growth advantage, allowing it to become the predominant cell type in the tumor (clonal expansion). Alternatively, the specific alteration detected might have arisen coincident with another, perhaps undetected, alteration that was the crucial change underlying clonal outgrowth.

By using epigenetic markers such as the inactivation of the X-chromosome, it has been established that all colorectal carcinoma and almost all adenomas—even very small adenomas of only three to four mm in maximal extent—are of clonal origin (i.e., are derived from a single precursor cell). It should be noted that a few studies have suggested that some adenomas may not be clonal at the earliest stages.

As noted previously, only about 5% of colorectal cancer cases are associated with defined highly penetrant cancer syndromes. The bulk of these cases are attributable to hereditary nonpolyposis colorectal cancer syndromes (HNPCCs), with another significant subset associated with the familial adenomatous polyposis (FAP) syndrome (Table 31-1). A few other syndromes contribute the remainder of the cases.

Adenomatous Polyposis Coli Gene: Gatekeeper in Familial Adenomatous Polyposis and Sporadic Cancer

Familial Adenomatous Polyposis

FAP is an autosomal dominant syndrome affecting about 1 in 8,000 individuals in the United States and accounting for less than 0.5% of colorectal cancers (17). Hundreds to thousands of adenomas

Table 31-1 Genetics of Inherited Colorectal Tumor Syndromes

Syndrome	Features Commonly Seen in Affected Individuals	Gene Defect
Familial adenomatous polyposis (FAP)	Multiple adenomatous polyps (>100) and carcinomas of the colon and rectum; duodenal polyps and carcinomas; fundic gland polyps in the stomach; congenital hypertrophy of retinal pigment epithelium (CHRPE)	<i>APC</i> (>90%)
Gardner syndrome	Same as FAP; also desmoid tumors and mandibular osteomas	<i>APC</i>
Turcot syndrome	Polyposis and colorectal cancer with brain tumors (medulloblastoma)	<i>APC</i>
Colorectal cancer and brain tumors (glioblastoma)	<i>MLH1</i> , <i>PMS2</i>	
Attenuated adenomatous polyposis coli (AAPC)	Less than 100 polyps, though marked variation in polyp number (from ≈5 to >1,000 polyps) seen in mutation carriers within a single family	<i>APC</i> gene (predominantly 5' mutations)
Hereditary nonpolyposis colorectal cancer (HNPCC)	Colorectal cancer without extensive polyposis; other cancers include endometrial ovarian and stomach cancer; occasionally urothelial, hepatobiliary, and brain tumors	<i>MSH2</i> <i>MLH1</i> <i>PMS1</i> <i>PMS2</i> <i>GTBP</i> <i>MSH6</i>
Peutz-Jeghers syndrome	Hamartomatous polyps throughout the gastrointestinal tract; mucocutaneous pigmentation; estimated 9- to 13-fold increased risk of gastrointestinal (GI) and non-GI cancers	<i>LKB1/STK11</i> (30%–70%)
Cowden disease	Multiple hamartomas involving breast, thyroid, skin, central nervous system (CNS), and GI tract; Increased risk of breast, uterus and thyroid cancer; risk of GI cancer unclear.	<i>PTEN</i> (85%)
Juvenile polyposis syndrome	Multiple hamartomatous/juvenile polyps with predominance in colon and stomach; variable increase in colorectal and stomach cancer risk; facial changes	<i>DPC4</i> (15%) <i>BMPR1a</i> (25%) <i>PTEN</i> (5%)
MYH associated polyposis (MAP)	Multiple adenomatous gastrointestinal polyps, autosomal recessive often associated with somatic K-Ras mutations	MYH

arise in the large bowel and rectum beginning in the second decade of life. Although only a fraction of the adenomas may progress to cancer, the lifetime incidence of colorectal cancer in untreated FAP patients approaches 100% with a mean age of diagnosis of 36 years, necessitating the prophylactic removal of the patient's colon early in adult life. Up to 25% of FAP cases seem to be caused by de novo germ-line mutations and therefore do not show the characteristic autosomal pattern of inheritance.

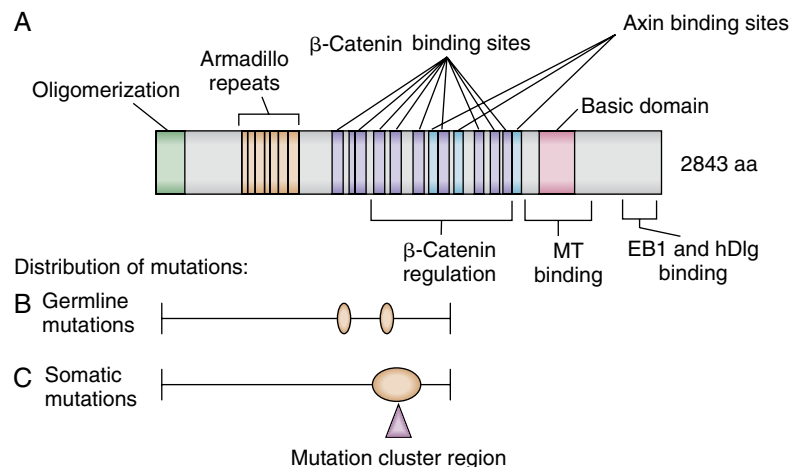
The gene that when mutated underlies FAP is the adenomatous polyposis coli (*APC*) tumor-suppressor gene on chromosome 5q21. Germ-line mutations have identified in one *APC* allele of affected individuals in 90% to 95% of families with FAP studied. More than 95% of mutations lead to premature truncation of *APC* protein synthesis—about two thirds by small insertions or deletions leading to a frame shift and the remainder by introducing a stop-codon (Figure 31-1). Several extracolonic tumors and symptoms are associated with FAP (Table 31-1). The combination of polyposis with brain tumors (in particular, medulloblastoma in pediatric cases) has been termed “Turcot syndrome” (19). Gardner syndrome comprises extensive polyposis with epidermoid cysts, desmoid tumors, and osteomas and seems to be correlated with mutations between *APC* codons 1403 and 1578 (20). Upper gastrointestinal polyps

are responsible for a large proportion of morbidity and mortality in patients after prophylactic total colectomy. This is particularly true for the duodenal cancers that develop in 4% to 12% of patients. Benign gastric fundic gland polyps and gastric adenomas, potential premalignant precursor lesions of gastric cancer, are observed at increased frequency, ultimately leading to stomach cancer in 0.5% of cases (21). In only a small percentage of FAP cases, thyroid cancer, bile duct cancer, hepatoblastoma (pediatric), and central nervous system tumors such as medulloblastoma are observed.

Somatic *APC* Mutations in Sporadic Tumors

Notwithstanding the critical role of the *APC* gene in FAP and related variant syndromes, the *APC* gene has an even more prominent role in sporadic colorectal tumors, as about 80% of sporadic colorectal adenomas and carcinomas have somatic mutations inactivating *APC* (22). *APC* mutations have been found in a number of the earliest sporadic lesions analyzed, including microscopic adenomas composed of only a few dysplastic glands (23). In addition to FAP, rare germ-line *APC* variants also seem to play a role in familial colorectal cancers of moderate penetrance (24). Epidemiologic studies revealed that

FIGURE 31-1 SCHEMATIC REPRESENTATION OF THE ADENOMATOUS POLYPOSIS COLI (APC) PROTEIN AND MUTATION HISTOGRAMS. **A:** Selected sequence motifs of the 2943-amino-acid APC protein and its interaction partners are indicated. The amino-terminal region has a domain that regulates its oligomerization. Repeated sequences with similarity to the *Drosophila* armadillo protein and its vertebrate homologue β -catenin (so-called armadillo repeats) are localized in the amino-terminal third of APC. Multiple independent 20-amino-acid repeats mediating binding to β -catenin and several binding sites for the Axin protein (termed “SAMP repeats”) are localized in the central third of APC. The carboxyl-terminal third contains a basic region that is involved in microtubule (MT) binding and mediates interactions with the proteins EB1 and hDlg. **B:** The frequency and distribution of germ-line mutations in familial adenomatous polyposis (FAP) patients are indicated with respect to the APC coding region. Virtually all mutations result in premature truncation at or very close to the mutation position. Two apparent mutational hotspots are seen at codons 1061 and 1309. **C:** Frequency and distribution of somatic APC mutations in sporadic colorectal cancers are indicated. The mutations appear to predominate in the “mutation cluster region” (MCR) and mutations at codons 1309 and 1450 are most common. (A–C from Ref. 18, with permission.)



the I1307K allele was present only in individuals of Ashkenazi Jewish origin, and those who carried the I1307K allele had a roughly twofold increased risk of developing colorectal cancer. As predicted by Knudson's two-hit model for tumor-suppressor genes, both APC alleles appear to be inactivated in colorectal adenomas and carcinomas by mutation of one allele and chromosomal loss of the remaining allele (22).

APC Protein Function

The APC tumor-suppressor gene encodes a large protein of approximately 300 kD that regulates cell–cell adhesion, cell migration, chromosomal segregation, and apoptosis in the colonic crypt (Figure 31-1; 25). Restoration of APC protein expression in colorectal cancer cells lacking endogenous APC expression promotes apoptosis (26). APC is known as a major binding partner and regulator of the β -catenin protein. β -catenin was first identified because of its role in linking the cytoplasmic domain of the E-cadherin cell–cell adhesion molecule to the cortical actin cytoskeleton, via binding to the adaptor molecule α -catenin. Based on elegant studies from many different investigators (reviewed in [27]) a model has been developed to explain the biologic significance of APC's interaction with β -catenin. The model proposes that in the absence of Wnt ligand signaling, APC binds and collaborates with the scaffold protein Axin to promote sequential phosphorylation by casein kinase I and glycogen synthase kinase-3 β (GSK3 β) of several conserved serine/threonine residues in the N-terminal region of β -catenin, thereby targeting β -catenin for ubiquitination and proteasomal degradation (Figure 31-2). In a physiological setting, the Wnt ligands inhibit degradation of β -catenin via binding to their cognate receptor complex of Frizzled proteins and LRP5/6 proteins.

In approximately 80% of colorectal cancers, both APC alleles are inactivated, virtually abolishing the coordinated destruction of β -catenin, essentially mimicking constitutive activation of Wnt ligand–mediated signaling (Figure 31-2). As a result, β -catenin accumulates in the cytoplasm, complexes with DNA binding proteins of the TCF (T-cell factor family)/Lef (lymphoid enhancer family) family, and translocates to the nucleus. Once there, β -catenin functions

as a transcriptional co-activator, activating the expression of TCF-regulated genes. In a subset of the cancers that lack mutations in APC, somatic mutations in β -catenin have been found (28). Further work then characterized β -catenin's role not only in the establishment of a crypt progenitor program but also in the spatial organization and migratory pattern of the cells in the continuous renewal of crypts. Strikingly, feedback inhibitors on several levels of the Wnt/APC/ β -catenin pathway seem to be among the most universal target genes of this pathway. Endogenous activation of this pathway by Wnt ligands is inhibited by WNT inhibitory factor-1 (WIF1) and Dickkopf-1 (DKK1). The intracellular signal transduction is inhibited by the homologues of the *Drosophila* gene naked and the product of the *Axin2* gene. Of interest, expression of these feedback inhibitors as well as the secreted–frizzled-related-protein (SFRP) family of extracellular inhibitors seems to be lost in early stages of colorectal carcinogenesis (29).

Mouse Models of FAP and Genetic and Epigenetic Modifiers

A mouse model of intestinal tumorigenesis known as the Min (for multiple intestinal neoplasia) mouse has been described (30). The Min mouse carries a germ-line mutation in the murine homologue of the APC gene, resulting in truncation of the murine *Apc* protein at codon 850. The intestinal tumor phenotype of Min mice is similar, but not identical, to that seen in FAP patients.

Other cellular genes can significantly influence the number of polyps that arise in mice heterozygous for the *Apc*^{Min} mutant allele or other mutant *Apc* alleles. For instance, when the *Min* mutation was introduced into mice with varying genetic backgrounds, significant variability in the number of intestinal tumors was seen (31). This finding was attributable to the effects of strain-specific modifier genes unlinked to the *Apc* locus. Other genes that substantially modify intestinal tumorigenesis in mice carrying a germ-line *Apc* mutation include genes encoding DNA methyltransferases. Specifically, mice that carry the *Min* mutation and a germ-line defect in one allele of the maintenance DNA methyltransferase *DNMT1* or a gut-specific deletion of both *DNMT3* alleles have a two- to threefold reduction in macroscopic

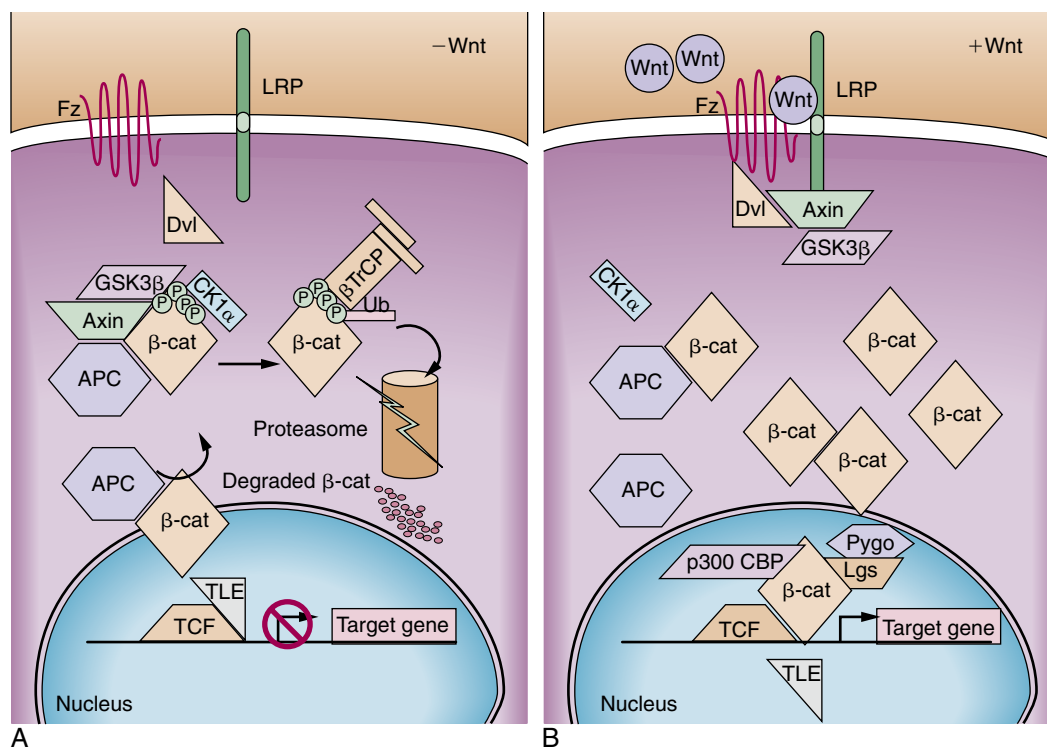


FIGURE 31-2 MODEL OF ADENOMATOUS POLYPOSIS COLI (APC) AND β -CATENIN FUNCTION **A:** The APC protein is part of a “destruction complex” containing glycogen synthase kinase 3β (GSK3 β), Axin and casein kinase 1α (CK1 α), which phosphorylates β -catenin in conserved serine and threonine residues in the N-terminus. This phosphorylation creates an epitope for recognition by the F-box protein β -TrCP as part of a ubiquitin ligase complex, which leads to polyubiquitination and proteasomal degradation of β -catenin. Hence, in most cells free β -catenin levels are kept low and transcription of target genes is repressed by recruitment of repressors of the transducin-like enhancer of split family (TLE). **B:** Upon binding of Wnt ligands to a cognate receptor complex consisting of one member of the Frizzled (Fz) family and one member of the low-density-lipoprotein receptor–related protein family (i.e., LRP5 or LRP6), the destruction complex is disassembled in part by recruitment of Axin to the LRP receptors and in part by not clearly defined action of disheveled proteins (Dvl’s). Similarly inactivating mutations of the APC protein or the Axin1 protein, or mutations of the conserved phosphorylation sites of β -catenin lead to the accumulation of free β -catenin which enters the nucleus and activates transcription of target genes by displacement of corepressors and recruitment of coactivators such as CBP/p300 and a complex of the homologs of *Drosophila* Legless (Lgs) and Pygopus (Pygo).

polyp number (32). Treatment of *Apc*^{Min} mice with 5-azacytidine, a pharmacologic inhibitor of DNA methyltransferases, resulted in a more than fivefold reduction in polyp number. Combining the genetic and pharmacologic manipulations of DNA methyltransferase activity synergistically reduced the polyp number roughly 50-fold. Treatment of mice with NSAIDs that inhibit COX-1 and COX-2 or agents that specifically inhibit COX-2 activity also markedly inhibited adenoma formation. The findings highlight the utility of the mouse genetic models for identifying novel genes and pharmacologic agents that modify intestinal tumor development.

Other Forms of Intestinal Polyposis

Other intestinal polyposis syndromes in which patients manifest numerous nonadenomatous lesions have been described. Several of these syndromes have an increased risk of gastrointestinal and/or nongastrointestinal cancers (Table 31-1). Starting from childhood, patients with the juvenile polyposis syndrome (JPS) develop multiple hamartomatous polyps throughout the gastrointestinal tract with some preference for the colon and the stomach. Colorectal cancers develop starting from an early age in about two thirds of JPS patients by the age of 60.

Cowden syndrome is an autosomal dominant syndrome in which affected individuals develop macrocephaly and hamartomas in many organ sites, including the breast, thyroid, skin, central nervous system, and gastrointestinal tract. The gene responsible for Cowden syndrome is the *PTEN* tumor-suppressor gene on chromosome 10q23 (33). Acting as a phospholipid-phosphatase, the *PTEN* protein is a major antagonist of the phosphatidylinositol-3-kinase (PI3K) survival pathway. In a small number of nonfamilial cases of juvenile polyposis lacking other features of Cowden syndrome, *PTEN* germ-line mutations have been reported. Peutz-Jeghers syndrome, a rare autosomal dominant condition affecting fewer than 1 in 25,000, is characterized by gastrointestinal hamartomatous polyps and mucocutaneous melanin deposition. The hamartomatous polyps contain essentially normal epithelial cells, but the mucosal components are arranged abnormally. Germ-line mutations in the *LKB1/STK11* tumor-suppressor gene on chromosome 19p can be seen in a significant fraction of cases (34,35). Inactivation of this tumor-suppressor gene leads to the hyperactivation of the mammalian target of Rapamycin (mTOR) pathway, which is responsible for integrating the nutritional supply with cell proliferation and growth. Homozygous mutations in the base-excision repair pathway gene *MYH* have been described as causing a autosomal recessive adenomatous polyposis syndrome (36),

which has been termed “MAP” for MYH-associated polyposis syndrome.

DNA Mismatch Repair Deficiency and Hereditary Nonpolyposis Colorectal Cancer Syndrome

HNPCC was arguably the first inherited cancer syndrome to be well described in the literature (37). In 1913, Warthin presented a particularly striking example of a three-generation family with HNPCC (38). Following Warthin’s lead, Lynch and others described kindreds with autosomal dominant patterns of colorectal cancer, not accompanied by extensive polyposis (39). In such families, proximal colon cancers of early onset were seen, along with cancers in some other organs including gastric, uterine endometrial, ovarian, renal, and hepatobiliary cancers (40).

Criteria for the clinical diagnosis of HNPCC include the Amsterdam II criteria (last revised in 1999) and the Bethesda criteria (last revised in 2004; 37). Also known as the 3–2–1 rule, Amsterdam II criteria specify affected families must show HNPCC-typical tumors of three relatives (one being a first-degree relative to the other two) in at least two successive generations, with one of the tumors occurring before age 50. The sensitivity of these criteria is 78% with a specificity of 46% to 68%. Linkage to chromosome 2p was seen in several kindreds with HNPCC (41). In other HNPCC families, the predisposition gene was localized to chromosome 3p (42). In yet other families with HNPCC, no evidence for linkage to chromosome 2p or 3p was found. The findings established that HNPCC was a genetically heterogeneous disease.

To explore Knudson’s two-hit hypothesis for HNPCC genes, investigators sought to demonstrate loss of the wild-type HNPCC allele on chromosome 2p in cancers from individuals carrying a defect in that particular predisposition gene. However, not only was there no loss of heterozygosity (LOH) of chromosome 2p sequences in the cancers, but microsatellite DNA sequences examined in this analysis demonstrated marked length variations in tumor tissues compared with the patient’s normal tissue. Microsatellite changes were seen at many different loci scattered throughout the genome and in all tumors from patients with HNPCC. The phenotype was termed the “microsatellite instability” (MSI) or “replication error” (RER) phenotype and cancers with evidence of microsatellite instability at more than 40% of a panel of mononucleotide and dinucleotide sequences are termed “high-frequency MSI” (MSI-H) cancers. Although most colorectal cancers display no instability when a panel of microsatellite tracts are studied—so-called microsatellite stable (MSS) cases—a subset of cancers show low-frequency instability of the microsatellite markers, termed “MSI-L.”

The finding of MSI-H in the cancers was noteworthy because similar DNA instability phenotypes had previously been seen in mutant yeast strains with defective DNA mismatch repair genes. The prediction that defects in one or more DNA mismatch repair genes might underlie the HNPCC syndrome was quickly borne out (Figure 31-3). A human homologue of the bacterial *mutS* mismatch repair gene, designated *MSH2*, was mapped to chromosome 2p and one allele was found to be mutated in the

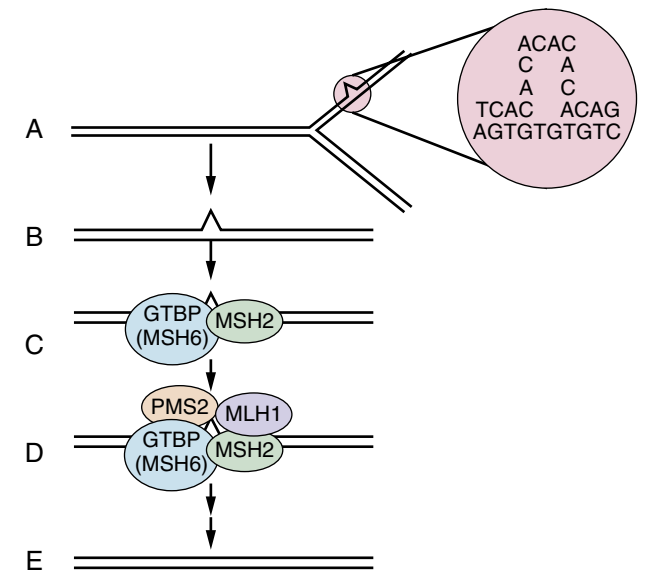


FIGURE 31-3 MISMATCH REPAIR PATHWAY IN HUMAN CELLS. A, B: During DNA replication, DNA mismatches may arise, such as from strand slippage (shown) or misincorporation of bases (not shown). C: The mismatch is recognized by MutS homologues, perhaps most often MSH2 and GTBP/MSH6, although MSH5 may substitute for GTBP/MSH6 in some cases. D, E: MutL homologues, such as MLH1 and PMS2, are recruited to the complex, and the mismatch is repaired through the action of a number of proteins, including an exonuclease, helicase, DNA polymerase, and ligase. (A–E from Ref. 21, with permission.)

germ-line of a subset of HNPCC patients, with the remaining allele inactivated in cancers arising in the mutation carriers. Other genes involved in DNA mismatch repair were studied and found mutated in other groups of HNPCC patients, including the *MLH1* gene on chromosome 3p, the *PMS1* gene on 2q, the *PMS2* gene on 7q, and the *GTBP/MSH6* gene on chromosome 2p. Mutations in the *MSH2* and *MLH1* genes are a far more common cause of HNPCC than mutations in the other mismatch repair genes, with *MSH2* and *MLH1* mutations together accounting for about 75% of the known mutations present in HNPCC patients (Table 31-2). About 70% of patients fulfilling the clinical Amsterdam II criteria lack identifiable mutations of the previously mentioned genes associated with HNPCC.

Table 31-2 Germ-Line Mismatch Repair Gene Mutations in HNPCC

Gene	Chromosome Location	<i>Escherichia coli</i> Homologue	Estimated % of HNPCC Families with Mutation
<i>MSH2</i>	2p15–16	MutS	≈35
<i>MLH1</i>	3p21	MutL	≈25
<i>PMS1</i>	2q31	MutL	unclear
<i>PMS2</i>	7p22	MutL	<3%
<i>GTBP/MSH6</i>	2p15–16	MutS	8%
Other/unknown	??	??	≈25–30*

HNPCC, hereditary nonpolyposis colorectal cancer syndromes; ??, not yet defined.
* Estimate is based on the fact that ≈93% of patients who had a family history meeting the Amsterdam Criteria displayed the MI/RER phenotype.
From Ref. 13 with permission.)

In the normal cells of a patient with HNPCC, DNA repair is rarely impaired, because the cells have a wild-type copy of the gene (i.e., the copy inherited from the nonaffected parent). However, during cancer development, the remaining wild-type allele is inactivated by a somatic mutation. Following inactivation of the wild-type allele, full mismatch repair activity is lost (Figure 31-3). Then, affected cells manifest a mutator phenotype and accumulate mutations in a much more rapid fashion. HNPCC is, therefore, a disease with more rapid tumor progression from a benign, initiated clone to frank malignancy (Figure 31-4).

Although germ-line mutations in the known mismatch repair genes have only been detected in 2% to 4% of colorectal cancer patients, more than 15% of all colorectal cancers display the MSI-H phenotype (44). This observation could suggest that germ-line and/or somatic mutations in mismatch repair pathway genes may be present in a substantial fraction of colorectal cancers, regardless of the patient's family history. A significant fraction of the cases among patients younger than 35 years of age may be due to new or unrecognized germ-line mutations (45). However, overall de novo germ-line mutations and somatic mutations in the known mismatch repair genes are found in only a small fraction of sporadic MSI-H colorectal cancers. Instead, the inactivation of *MLH1* via hypermethylation of its promoter seems to be responsible for most MSI-H colorectal cancer cases outside of HNPCC families (44,46). Many of the mutations that arise in cells with the MSI-H phenotype may be detrimental to cell growth or may exert no positive selection pressure. However, a subset of mutations could potentially activate oncogenes or inactivate tumor-suppressor genes. Activating mutations in β -catenin seem to be a major factor in canonical Wnt signaling pathway activation in MSI-H cases (28).

An example of a gene containing a mononucleotide tract in its coding sequence and specifically inactivated in colorectal cancers of the MSI-H phenotype is the transforming growth factor β (TGF- β) type II receptor. Other genes altered in MSI-H

colorectal cancers are those encoding the apoptotic regulators Bax and caspase-5.

In addition to the disruption of the DNA mismatch repair in MSI-H colorectal cancer cases, alteration of the *MYH* base excision repair gene has been detected in patients with multiple colorectal adenoma and overt adenomatous polyposis (termed MAP for MYH-associated polyposis). Homozygous germ-line mutations of the *MYH* gene prevent the repair of oxidative damage of DNA thereby leading to increased GC to AT pair transversions (36). Interestingly, a large percentage of tumors in MAP patients show *K-RAS* mutations.

Inflammation and Colon Cancer

Chronic inflammatory bowel disease (IBD), especially UC, confers a high risk of colorectal cancer, perhaps only surpassed by the risk associated with FAP and HNPCC. In the case of UC, colorectal cancer risk is associated with both the duration and extent of the inflammatory disease. From a pathogenetic standpoint it is notable that UC-associated cancers often develop without the formation of a polyp as a precursor lesion, although dysplasia is a common factor (15). Reducing the degree of inflammation in IBC, such as by mesalazine treatment, seems to reduce the risk for colorectal carcinoma (47). The pathways by which inflammation supports colorectal carcinogenesis are not well understood.

The response of the immune systems to normal intestinal flora seems to modulate the development of intestinal neoplasia. Interleukin-10 knock-out mice and mice carrying homozygous mutations in interleukin-2 and β_2 -microglobulin develop spontaneous enterocolitis and adenocarcinoma, and intestinal flora can substantially modulate phenotype. Disruption of TGF- β signaling pathway components, such as the essential SMAD downstream signaling proteins, also seems to regulate intestinal tumorigenesis via the immune system. Homozygous disruption of the *SMAD3* gene in mice was initially described to predispose to colorectal adenocarcinoma. Defects of the *SMAD4* gene, encoding a SMAD3-interacting protein, are also seen in some human colorectal cancers. Interestingly, TGF- β knock-out mice on an immunodeficient background also show a gut-flora-dependent development of colorectal carcinoma (48).

Whereas the findings described in the preceding sections have offered support for the notion that the immune system response to gut flora plays an essential role in tumorigenesis, some other findings have implicated the response of the intestinal epithelium itself to the gut flora in tumorigenesis (45,49,50).

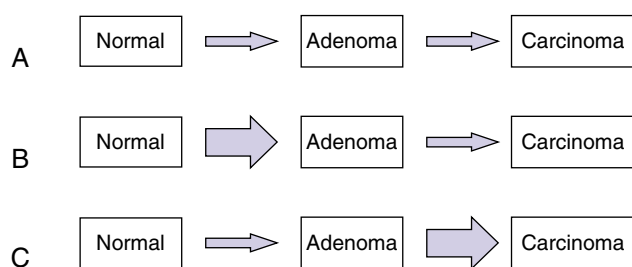


FIGURE 31-4 RELATIVE EFFECTS OF GERM-LINE MUTATIONS ON TUMOR INITIATION AND PROGRESSION A: In sporadic cancers, both initiation of a neoplastic lesion (e.g., the adenoma) and progression to an advance lesion (i.e., the carcinoma) are rate-limiting events because two somatic mutations are required for inactivation of tumor suppressors such as adenomatous polyposis coli (APC) (initiation of adenoma) and p53 (tumor progression). **B:** In the case of familial adenomatous polyposis (FAP), germ-line inactivation of one APC allele markedly increases the formation of adenomas, because inactivation of both APC alleles is a critical (rate limiting) event in adenoma formation, and those with inactivation of one allele in all colonic cells need only inactivate the remaining APC allele to initiate adenoma formation. **C:** In the case of hereditary nonpolyposis colorectal cancer (HNPCC), germ-line inactivation of one of the mismatch-repair genes (e.g., *MSH2* or *MLH1*) coupled with somatic inactivation of the remaining allele in an initiated lesion (e.g., an early adenoma) greatly increases the mutation rate, and subsequently the rate and speed of progression to more advanced lesions. (From Ref. 43, with permission.)

Common Somatic Alterations in Colorectal Cancer

Somatic alterations play a pivotal role in carcinogenesis. A sizeable number of mutational alterations in colorectal cancer have been identified. However, only in the recent past, have largely unbiased sequencing analyses of a large fraction of genes permitted a fuller view of the nature and spectrum of mutational alterations in colorectal cancers (16).

Oncogene Activation

K-Ras, B-Raf, and PIK3ca Mutations

The RAS family of small G-proteins encodes molecular switches that function in several growth factor signaling pathways. Its three members—*K-RAS*, *H-RAS*, and *N-RAS*—are frequently altered by somatic mutations in various human and animal tumor types. Mutations in the *K-RAS* genes can be identified in about 40% of colorectal carcinomas (51, 52). The vast majority of *ras* mutations are present at codons 12 and 13 of *K-RAS*, with about 70% of the *ras* mutations present at codon 12 and about 20% present at codon 13. Evidence suggests that *K-RAS* mutations may contribute to progression of colorectal adenomas but are not required for initiation of adenoma formation. In adenomatous polyps, the frequency of *K-RAS* mutations is clearly dependent on the size and the dysplasia of the lesion. Only 10% of adenomas smaller than 1 cm show *K-RAS* mutations, whereas *K-RAS* mutations are observed in about 50% of adenomas larger than 1 cm. A small fraction of *RAS* gene mutations are present at codon 61 of *K-RAS* and codons 12, 13, and 61 of *N-RAS*. No *H-RAS* mutations have been described in colorectal tumors. The biologic basis for the particular spectrum of *ras* mutations seen in colorectal tumors is still subject to debate.

Genetic strategies, resulting in disruption of the mutant *K-RAS* alleles in advanced colorectal cancer cells, have demonstrated that inactivation of mutant *K-RAS* activity abrogated the tumorigenic growth properties of the cells in *in vitro* and animal studies. *RAS* proteins are major regulators of several downstream signaling cascades. It is therefore not surprising that other components in these pathways are mutated in a fraction of colorectal cancers. *B-RAF*, a protein kinase directly activated by *RAS* proteins and which in turn activates the mitogen-activated protein kinase

(*MAPK*) family, is mutated in about 5% of all colorectal cancers. *Ras* proteins can also activate the phosphatidylinositol-3-phosphate pathway, resulting in the downstream activation of protein kinase *B/AKT* with resultant activation of downstream anti-apoptotic factors and the *mTOR* pathway, which integrates nutrient availability with cellular growth. The formation of phosphatidylinositol-3 phosphate is dependent on the catalytic activity of the gene product of the *PIK3CA* gene. Mutations of this gene are observed in between 13% and 30% of colorectal cancers. Subsequent studies showed that these mutations activate the kinase activity (53).

Other Oncogene Alterations in Colorectal Tumors

Besides *K-RAS*, *B-RAF*, and *PIK3CA*, specific alterations in other cellular oncogenes have not been detected in a high percentage of colorectal tumors of any stage (Table 31-3). As described in the section on *APC* function, point mutations and small in-frame deletions in the amino-terminal sequences of β -catenin are present in about 2% to 5% of colorectal cancers (28). Certain proto-oncogenes have been found to be amplified in a very small percentage (i.e., <5%) of the cases studied. Among the proto-oncogenes affected by gene amplification are *NEU* (also known as *HER2* or *ERBB2*), *C-MYC*, *MYB*, cyclin D1, and cyclin E. In the recent systematic large-scale sequencing approaches in colorectal carcinoma, several other candidate proto-oncogenes have been found to be mutated in a small fraction of cancers (16).

Inactivation of Tumor-Suppressor Genes

Some of the earliest evidence indicating that tumor-suppressor gene mutations might be common in colorectal tumors was obtained from studies of allelic losses (54). Loss of a chromosome region in the tumor cells is often termed an “allelic loss” or “loss

Table 31-3 Somatic Mutations in Oncogenes and Tumor-Suppressor Genes

Gene	Type of Mutation	Frequency of Alterations
Oncogenes		
<i>K-RAS</i>	Point mutation (codons 12, 13, 61)	40% (>75% of mutations at codon 12)
<i>N-RAS</i>	Point mutation (codons 12, 13, 61)	<5%
<i>PIK3</i>	Point mutations activating kinase activity	14%–35%
β -catenin	Point mutation and in-frame deletions of amino terminal	~2%–5%
GSK3 phosphorylation sites		
<i>NEU (HER-2/ERBB-2)</i>	Amplification	<5%
<i>C-MYC</i>	Amplification	<5%
<i>MYB</i>	Amplification	<5%
Tumor-suppressor genes (and candidate tumor-suppressor genes)		
<i>p53</i>	Point mutation, LOH	>60%; >95% of pt mutations are missense
<i>APC</i>	Small insertion or deletion, or nonsense with premature truncation of protein synthesis	>70%; >95% of mutations generate frame-shift point mutation, LOH
<i>DPC4/SMAD4</i>	Point mutation, LOH	LOH in ~60%; missense and nonsense mutations in ~10%–15%; homozygous deletions in ~2%
<i>SMAD2/JV18-1</i>	Point mutation or small deletion, LOH in <5%	LOH in ~60%; missense mutations or small deletions
<i>NF-1</i>	Point mutation	Unknown
<i>TGF-βRII</i>	Small insertion or deletion, in mononucleotide repeat point mutation	10%–15%; only cancers with microsatellite instability (100%), premature translational termination
<i>DCC</i>	Insertion, deletion, LOH in ~10%–15%; homozygous deletions in ~2%	LOH in ~60%; microsatellite insertions in intron
<i>MCC</i>	Point mutation, LOH	LOH in ~50%; localized inactivating mutations in ~5%

LOH, loss of heterozygosity; gene abbreviations are described in the text.

of heterozygosity" (LOH). In the allelotype of colorectal cancers, certain chromosome arms have been found to be more frequently affected than others, such as 5q, 8p, 17p, and 18q. Although the allelotype pattern for colorectal cancer as a whole appears complex, individual tumors have a less complex pattern (55). Allelotype studies of colorectal cancers have yielded data generally consistent with those from karyotypic analyses. Both approaches likely underestimate the prevalence and complexity of tumor-suppressor gene alterations, because more localized mutations in tumor-suppressor genes cannot be detected by gross chromosome survey methodologies, like those in allelotype and karyotype studies. DNA microarray-based comparative genomic hybridizations have been instrumental in identifying regions of allelic imbalance where copy number is actually increased, such as 20q and 8q (55). Presumptive tumor-suppressor genes on chromosomes 5q, 17p, and 18q have been identified.

Genetic Instability: Chromosomal Instability versus DNA Mismatch Repair Deficiency

In allelotype studies, it was found that 10% to 15% of colorectal cancers appeared to suffer no allelic losses (Figure 31-5; 2). We now know that the cancers with very few or no allelic losses, in fact, display the MSI-H phenotype. Key factors underlying chromosome instability are rather poorly defined, but some clues have emerged. Systematic DNA sequencing approaches have been performed focusing on genes whose homologues have been shown to be involved in proper chromosome segregation and M-phase checkpoints in other systems. This led to the identification of three groups of genes that are altered in cancers with chromosomal instability. These represent genes involved in double-strand break repair (e.g., *MRE11*) and proper chromosome segregation (e.g., *DING*, *hRod*, *hZwilch*, *hZw10*) at a low rate. Inactivation of the tumor-

suppressor gene APC alone seems to confer a certain chromosomal instability.

Epigenetic Changes in Colorectal Carcinogenesis: the CpG Island Hypermethylation Phenotype

In mammalian genomes, most 5'-CpG-3' dinucleotides have been lost during evolution. DNA methylation covalently modifies more than 80% of the remaining CpG sites, except for localized regions of high CpG-dinucleotide content, which have been termed "CpG islands." The promoters of about 50% of all genes contain CpG islands. Hypermethylation of these CpG islands seems to be associated with transcriptional silencing of downstream transcriptional units, perhaps reflecting an epigenetic mechanism to reinforce long-term gene silencing following more transient chromatin modifications (56). Although the global trend in colorectal cancer cells is hypomethylation, the CpG island hypermethylation of several promoters shows increased methylation with ensuing transcriptional silencing (i.e., hypermethylation; 57). A large fraction of colorectal cancers show hypermethylation and transcriptional silencing of potential tumor-suppressor genes, such as *HIC1* and the Wnt-signaling antagonists *SFRPs* (29,58).

Tumors with global hypermethylation changes fit the model of the CpG island hypermethylation phenotype (CIMP), and distinct markers of this phenotype have been suggested (46,59). A subset of CIMP tumors shows hypermethylation of the *MLH1* mismatch repair gene, and they are the major fraction of apparently sporadic MSI-H tumors. This subset of MSI-H cases often harbors gain-of-function mutations in *B-RAF*. B-Raf mutations are commonly observed in sessile serrated adenomas (SSAs) and the cancers presumed to arise from SSAs (13, 14). Whereas a role for hypermethylation in colorectal cancer development has been clarified to some extent through studies of the genes apparently silenced in part via promoter hypermethylation, knowledge about the contribution of DNA hypomethylation to the cancer process is less clear.

Chromosome 17p and the *p53* Gene

One of the chromosome regions commonly affected by LOH in colorectal cancers is chromosome 17p (54). Allelic losses of 17p are observed in about 75% of colorectal cancers, but are infrequent in adenomas, including large, late-stage adenomas. The common region of LOH on 17p includes the *p53* gene. Sequence analysis of the remaining *p53* allele from a large number of colorectal carcinomas with 17p LOH revealed that *p53* missense mutations were present in the overwhelming majority of cases (60). Adenomas without 17p LOH rarely have *p53* mutations, although the few adenomas with 17p allelic losses have been found to have *p53* mutations. Although the high mutation frequency of *p53* suggests that its inactivation might be the major force underlying selection for 17p LOH in colorectal cancer, other genes might also be inactivated by 17p LOH. For instance, the remaining allele of the hypermethylated in cancer-1 (*HIC1*) gene seems to be inactivated epigenetically. In light of the tumor predisposition observed in mice carrying heterozygous inactivating mutations in *HIC1*, bi-allelic *HIC1* inactivation could represent an additional driving force for 17p loss (61).

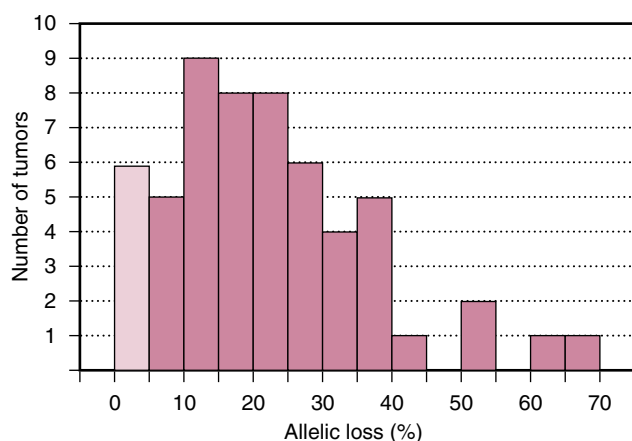


FIGURE 31-5 FREQUENCY OF ALLELIC LOSSES IN COLORECTAL CARCINOMA. DNA markers from all nonacrocentric autosomal chromosomes were used to determine the frequency of allelic losses. The percentage allelic loss is derived by dividing the number of allelic losses by the number of chromosomal arms that were evaluable (i.e., informative) in the allelotype analysis and expressing the frequency as a percentage. Of the 60 colorectal carcinomas analyzed, the percentage allelic loss varied from 0% to nearly 70%, with the median of 20%. The six cancers (i.e., 10% of the group of 60 cancers) that had no detectable allelic losses (indicated by the hatched bar) have the microsatellite instability (MSI) or replication error (RER) phenotype. The vast majority of cancers display the chromosomal instability phenotype. (From Ref. 52, with permission.)

The *p53* mutations in colorectal tumors have been found primarily in the most conserved regions of the gene, similar to data from other human cancers. Codons 175, 248, and 273 seem to be most frequently involved in colorectal tumors. Although *p53* defects play a significant role in colorectal cancer development, the *p53* mutations arise preferentially at later stages of tumor development. Consistent with this notion, germ-line *p53* mutations, such as those present in individuals with the Li-Fraumeni syndrome, do not increase the risk of colorectal cancer appreciably. The biological basis for *p53* inactivation preferentially in later stage colorectal lesions is unknown.

Chromosome 18q and the *DCC*, *DPC4/SMAD4*, and *SMAD2* Genes

LOH of chromosome 18q can be seen in about 70% of colorectal carcinomas; approximately 50% of large, late-stage adenomas; and less than 10% of early-stage adenomas (54). Three different genes from the region of chromosome 18q affected by LOH have been suggested as targets for inactivation in colorectal tumors. These genes are *DCC*, *DPC4/SMAD4*, and *SMAD2*. Unfortunately, conclusive evidence that one or more of these genes is a common and crucial target of inactivation in colorectal cancers is still lacking because frequent intragenic somatic mutations have not yet been found in any one of the genes in primary colorectal cancers.

The *DCC* gene is extremely large, spanning greater than 1.3 million base pairs, and it contains 29 or more exons (62). The *DCC* protein is a transmembrane protein, and, together with related proteins from the *UNC5* family, *DCC* represents a receptor for the netrin-1 protein. To explain the potential role of *DCC* inactivation in tumorigenesis, *DCC* has been proposed to function as a dependence receptor, a class of molecules that promotes apoptosis in the absence of ligand binding. Support for this model is provided by the observation that transgenic animals with intestinal overexpression of netrin-1 develop higher grade and more advanced lesions in cooperation with *Apc* inactivation (63).

Definitive evidence supporting a causal role for *DCC* alterations in colorectal tumors has largely been lacking. However, there is evidence to support the proposal that *DCC* is a candidate tumor-suppressor gene. One *DCC* allele is affected by loss in upwards of 70% of colorectal cancers. Although low levels of *DCC* expression have been detected in most normal adult tissues, including colonic mucosa, *DCC* gene and protein expression have been found to be markedly reduced or absent in more than 50% of primary colorectal cancers and cell lines. Loss of *DCC* expression has been associated with progression from adenoma to carcinoma in individual tumor specimens containing both components, and *DCC* loss of expression seems to be correlated with poor outcome in patients with colon cancer. Somatic mutations in the *DCC* gene have been detected in only about 15% of colorectal carcinomas. Like many microsatellite alterations in colorectal cancer, insertions in the *DCC* microsatellite element are found in MSI-H cancers. The insertions are clonal, present in all of the neoplastic cells, and associated with reduced or absent *DCC* expression (64).

A compelling chromosome 18q tumor-suppressor gene in colorectal cancers is the *SMAD4* gene. The *SMAD4* gene was first found to be affected by homozygous deletion or more localized

mutations in about 40% to 50% of pancreatic cancers, or roughly half of the pancreatic cancers with 18q LOH. The *SMAD4* protein mediates downstream TGF- β signaling events via its function as a transcription factor. Because of the fact that many colorectal cancers display insensitivity to the growth inhibitory effects of TGF- β as well as the location of the *SMAD4* gene in the common region of LOH on 18q, detailed studies of *SMAD4* alterations in colorectal cancers have been undertaken. Somatic *SMAD4* mutations have been detected in about 10% to 15% of colorectal cancers (66). The *SMAD2* gene is located in the same vicinity of *SMAD4* on chromosome 18q, and somatic mutations in *SMAD2* are present in 5% or less of colorectal cancers. In light of the function of the *SMAD2* and *SMAD4* proteins as downstream mediators of TGF- β signaling and somatic mutations are found in the genes in some colorectal cancers, it seems clear that *SMAD2* or *SMAD4* inactivation has a contributing role in colorectal cancers. Nevertheless, because *SMAD4* and *SMAD2* are inactivated in only a fraction of the 70% of colorectal cancers with 18q LOH, neither gene is the principle target of chromosome 18q LOH in colorectal cancer.

Multistep Genetic Models of Colorectal Tumor Development

Based on the oncogene and tumor-suppressor gene alterations reviewed previously, a genetic model of colorectal tumorigenesis has been proposed (65). The model relies on the assumption that most carcinomas arise from preexisting adenomas, and a modified version of the model for the roughly 85% of tumors displaying chromosomal instability is presented in Figure 31-6. Each of the genetic alterations described in the model is seen at high frequency only at particular stages of tumor development. Hence, this is the basis for assigning a relative order to the alterations in the multistep pathway. However, the order of the mutations is not invariant, as small adenomas with *p53* mutations have been identified and *K-ras* mutations can be associated with progression to carcinoma in some late-stage adenomas.

Other distinct molecular pathways to colorectal carcinogenesis have been suggested, and two alternative scenarios are presented in Figure 31-6. The approximate 2% to 4% of cases arising in the setting of germ-line mutations of DNA mismatch repair (MMR) genes likely share some similarities in the lesions that give rise to adenomatous lesions, such as *APC* and *K-RAS* mutations. In the cases of the approximate 10% to 12% of apparently sporadic cancers that show the MSI-H phenotype, many of the cancers presumably arise from serrated adenomatous lesions and some of the molecular lesions associated with the genesis of the serrated adenomas and the subsequent progression to carcinoma are distinct from those in the CIN and HNPCC-type MSI-H lesions, including frequent *B-RAF* mutations and the silencing of certain tumor-suppressor genes in part via promoter hypermethylation. Finally, though not depicted in Figure 31-6, it is worth noting that the nature and order of mutational events seems to be different in UC-associated cancers than in sporadic cancers. For instance, *p53* mutations are typically observed at an earlier

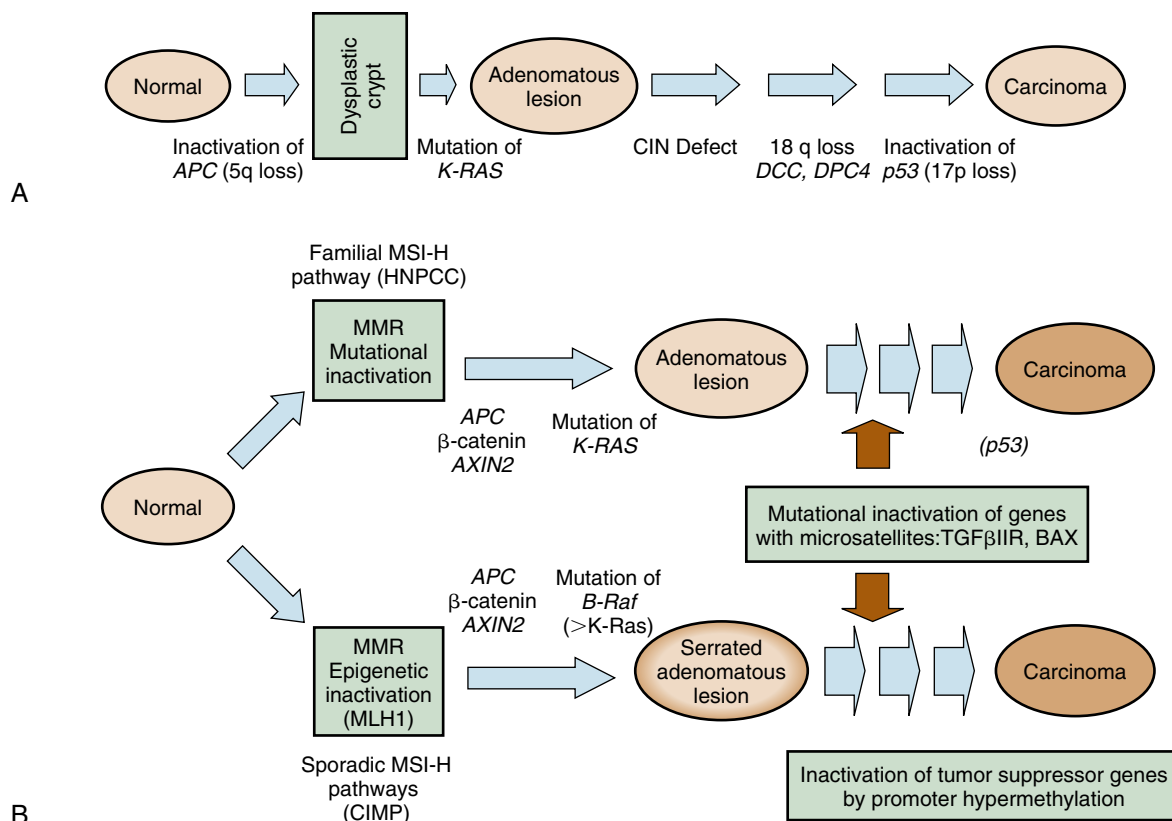


FIGURE 31-6 GENETIC MODEL OF COLORECTAL CANCER. A: Most colorectal cancers are believed to arise from adenomatous polyps over a period of years or even decades. The inherited and somatic genetic alterations believed to underlie tumor initiation and progression are indicated and discussed in detail in the text. Although their order is not invariant, the mutations show strong association with particular stages of tumorigenesis. (From Ref. 65, with permission.) **B:** In about 20% of colorectal cancers mismatch repair (MMR) function is inactivated either by somatic mutations or by epigenetic inactivation leading to microsatellite instability (MSI-H). Mutational inactivation of MMR genes is most commonly observed as second hit in patients that already carry germ-line mutations in MMR genes and fall under the hereditary nonpolyposis colorectal cancer syndrome. Epigenetic inactivation of MMR genes most commonly affects hypermethylation of the MLH1 promoter. These tumors often initially present as serrated adenomatous lesions, show hypermethylation of numerous genes (CpG island methylation phenotype, CIMP) and show *B-Raf* mutations. In both familial and sporadic MSI-H tumors, inactivation of genes with repetitive elements (microsatellites) in their coding sequence might contribute to tumor progression (e.g., *TGFβIIIR*, *BAX*).

time point, perhaps even occurring in the nonneoplastic inflamed mucosa of some patients (67).

Clinical Applications of Molecular Genetic Insights

The advances in our understanding of the inherited and somatic genetic alterations in colorectal cancers have made possible clinical applications that should improve the diagnosis and care of patients and families affected by colorectal cancer. Although many possible future clinical applications can be envisioned, only a few that are being actively pursued will be described in the following sections.

Risk Assessment

The accurate presymptomatic diagnosis of FAP or HNPCC is of significant value to members of families with these syndromes. The identification of germ-line mutations in the *APC* gene in more than 80% of families with FAP and Gardner syndrome provides the basis for genetic counseling of families at risk for polyposis (17,36). NSAIDs, such as COX-2 inhibitors, show activity in inhibiting colon adenoma development and progression in the setting of FAP (68).

Early Detection

The results of clinical trials indicate that the colonoscopic removal of larger (>1 cm) adenomas and early colorectal carcinomas has a major impact on colorectal cancer incidence and very likely on mortality for colorectal cancer (7). The development of highly specific and sensitive molecular tests for early detection of colorectal cancer is an important goal, given the reduced specificity and sensitivity of current noninvasive tests, such as fecal occult blood testing (1,36). If inexpensive and reliable molecular diagnostic tests of stool specimens could be developed, such tests might serve an adjunctive role along with more invasive and expensive methods for early detection, such as colonoscopy.

Findings from studies of DNA isolated from stool samples of patients known to have carcinomas or large, advanced adenomas indicate that stool-based tests for mutant oncogenes and tumor-suppressor genes may have utility (69). Using a multitarget DNA assay panel (MTAP), consisting of 21 localized mutations in the *K-ras*, *APC*, and *p53* genes, along with instability at a microsatellite repeat marker known as BAT-26, studies have been pursued to assess the sensitivity and specificity of the MTAP for detection of clinically significant lesions in asymptomatic individuals (69,70). The fecal DNA MTAP detected 16 of 31 invasive cancers, whereas Hemocult II

only identified 4 of 31 (51.6% vs. 12.9%, $p = 0.003$). The fecal DNA MTAP detected 29 of 71 invasive carcinomas plus adenomas with high-grade dysplasia, whereas the Hemoccult II test identified 10 of 71 (40.8% vs. 14.1%; $p < 0.001$). Specificity of the fecal DNA MTAP was found to 94.4% (70). Clearly, although most clinically significant lesions were not detected by either the fecal DNA test or the fecal occult blood test, the fecal DNA MTAP had much higher sensitivity than the only currently recommended noninvasive screening test for colorectal cancer, without any reduction in specificity.

Prognostic and Predictive Markers

In addition to presymptomatic diagnosis (risk assessment) and early detection of tumors, several studies indicate that characterization of the specific genetic alterations in a cancer may provide improved/increased prognostic information about the likelihood of local and distant tumor recurrence. Perhaps the most robust prognostic markers defined thus far for colorectal cancer are those for the MSI-H phenotype. In particular, MSI-H phenotype has been convincingly associated with improved survival in stage II and stage III colorectal cancer patients (71). Interestingly, the use of 5-fluorouracil (5-FU)-based adjuvant chemotherapy did not appear to show any benefit in survival for patients MSI-H tumors (72). In fact, although not a statistically significant result in the initial study, the trend was for poorer survival in 5-FU-treated patients whose tumors displayed the MSI-H phenotype.

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Summary and Future Directions

Molecular genetic studies of colorectal tumors have yielded profound insights into inherited predispositions to colorectal cancer as well its pathogenesis. A relatively limited number of oncogenes and tumor-suppressor genes—the KRAS, APC, and p53 genes—have been found to be recurrently mutated in colorectal tumors, and intensive studies of the function of these critical genes in normal and neoplastic cell growth continue. The relative significance to the cancer cell phenotype of each of the various inherited and somatic mutations has not been well defined. Comprehensive sequence analyses also suggest that it is likely that considerable number of additional oncogenes and tumor-suppressor genes with roles in subsets of colorectal cancer remain to be identified. Identification of these genes and characterization of their contribution to cancer will be an important, albeit a challenging, task. At present, there is little understanding of the relationship between dietary and environmental agents associated with increased risk of colorectal cancer and the mutation rate and nature of the mutations that arise in normal and neoplastic cells in the colon and rectum. Only limited insights have been obtained into the true significance and generality of the findings from the clinical correlative studies undertaken to date. Nevertheless, it is clear that further efforts will yield insights into the molecular basis of colorectal cancer and can be expected to result in advances in the diagnosis and clinical care of patients with colorectal tumors.

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Breast Cancer

Adenocarcinoma of the breast is frequently studied and reported as a distinct entity, although as I discuss throughout the course of this chapter, in many respects breast cancer is quite diverse and heterogeneous. Estimates from the American Cancer Society are that in 2007 approximately 178,480 women in the United States will receive the diagnosis of invasive breast cancer, and an additional 62,030 women will be diagnosed with ductal carcinoma in situ (DCIS; 1). According to data from the Surveillance, Epidemiology, and End Results (SEER) program from 1975 through 2002 there has been a notable annual increase in breast cancer incidence up until 1998, at which point incidence began to decrease by approximately 1% per year. Analysis of 2003 data from the SEER program has called attention to an apparent 7% decrease in breast cancer incidence in the United States in that year alone. A possible link to nation-wide reductions in the use of postmenopausal hormonal replacement therapy was suggested by the observation that this marked decrease was most notable in women older than 50, and more dramatic for cancers that expressed the estrogen receptor (ER; 2). It should be noted, however, that significant reductions in prescriptions for estrogen–progestin combination hormone therapy only followed the early termination of the estrogen–progestin treatment arm of the Women's Health Initiative in the late spring of 2002 and publication of findings of its adverse effects later that summer. The coming years will clarify whether this decrease in breast cancer incidence is progressive and sustained or limited and transient.

Biology of Breast Cancer Risk

Hormones

That an individual's risk of breast cancer is primarily influenced by hormonal factors is clear from many epidemiologic and intervention studies, most notably by the fact that the ratio of breast cancer incidence in women to men is 125 to 1 (1). Endogenous levels of estrogen in women appear to play a role in determining risk. A woman's lifetime exposure to endogenous estrogen is multifactorial, including overall nutrition during youth and its impact on age of menarche, and postmenopausal obesity, which results in an increase level of tissue aromatase and thus greater conversion of

androstenedione to estrone. Exogenous hormones, especially the combination of estrogen plus progestin, very commonly prescribed to postmenopausal women until recently, is also associated with an increased risk of breast cancer (3).

The actions of estrogen in breast tissue are varied. Perhaps most studied are actions mediated by the originally described estrogen receptor ($ER\alpha$). Binding of estradiol to the $ER\alpha$ leads to its translocation to the nucleus, dimerization, and binding to estrogen-responsive elements in regions of DNA that regulate expression of a range of proteins that collectively promote growth of mammary epithelium. Many of the downstream consequences of estrogen signaling affect cell cycle, apoptosis, and cell motility by elaboration of peptide growth regulatory proteins, some of which function in both autocrine and paracrine modes. Among these downstream mediators of estrogen action are epidermal growth factor (EGF) and its ligand amphiregulin, transforming growth factors α and β (TGF- α and TGF- β), platelet-derived growth factor (PDGF), and the insulin-like growth factors (IGF-I and IGF-II). One of the genes whose expression in mammary epithelium is induced by the action of the $ER\alpha$ is the progesterone receptor (PR), and its expression with $ER\alpha$ in breast cancer is an indicator of continued hormonal responsiveness. Estrogen receptor-mediated signaling is now understood to be more complex with the identification of a second receptor, $ER\beta$ (3). These two forms of ER bind to various ligands with different affinities and probably exhibit differences in recruitment and interaction with transcriptional coactivators. The role of $ER\beta$ in breast cancer progression, treatment, and prevention via anti-estrogen therapy has yet to be elucidated.

Studies have also documented the presence of membrane-bound forms of ER that can bind to estrogen and generate non-transcriptional effects on a rapid timescale. These effects include activation of mitogen-activated protein kinases and changes in cyclic adenosine monophosphate (AMP) levels (3). The potential consequences and opportunities for prevention and therapy that stem from this nontraditional action of the ER, interfacing with other signal-transduction pathways, has yet to be fully explored.

Estrogen can promote carcinogenesis by receptor-independent mechanisms as well. Oxidative metabolism of estrogen by cytochrome P-450 enzymes yield hydroxycatechol and semiquinones, which can lead to DNA mutations by direct covalent binding as well as via generation of reactive oxygen species.

Heritable Factors

Inherited predisposition to breast cancer represents a significant proportion to the overall incidence of the disease. A landmark study on the importance of heritable factors on cancer incidence was based on long-term twin registries in Sweden, Denmark, and Finland in which the incidence of various cancers among 44,788 pairs of twins was analyzed, comparing monozygotic and dizygotic twins. From that analysis it was estimated that approximately 25% of breast cancers could be attributed to heritable factors (4).

The most common syndrome of inherited predisposition to breast cancer was identified as an autosomal dominant, highly penetrant genetic syndrome that significantly increases the risk of both breast and ovarian cancer. From linkage analyses with families exhibiting this syndrome two distinct genes, *BRCA1* and *BRCA2*, have been discovered. Pathogenic mutations in *BRCA1* and *BRCA2* are now routinely tested for as possible genetic risk factors in young women with breast and/or ovarian cancer, especially in association with a strong family history of these cancers. The age-specific penetrance of inherited mutations in these genes, although different for the two genes and the two cancers, is notable for a marked shift toward younger age of diagnosis. These genes exhibit multiple biologic functions, and the specific activities that underlie their role as suppressors of breast carcinogenesis have yet to be firmly established, although evidence suggests that regulation of gene expression and facilitation of DNA repair are important aspects of their overall function as tumor-suppressor genes. It is important to note that whereas breast cancers that typically arise in women who harbor mutations in *BRCA1* lack expression of the ER, breast cancers that develop as a consequence of a *BRCA2* mutation are usually of the ER-positive phenotype (5).

The other genes that are highly penetrant tumor-suppressor genes in breast cancer (i.e., result in a very significant increase in risk when a mutant copy is inherited) are much less prevalent in the population and therefore somewhat less clinically relevant overall. They include *p53* (Li-Fraumeni syndrome), *PTEN* (Cowden syndrome), and *LKB1/STK11* (Peutz-Jeghers syndrome; 6–8). In contrast to these infrequently mutated yet highly penetrant genes, there are likely to be a number of genes more commonly mutated in the population that result in a modest increase in breast cancer risk. Perhaps the best studied gene in this category is *CHEK2*, which plays a role in cellular response to DNA double-strand breakage. In a population-based study from Denmark, carriers of a common deletion mutation in *CHEK2* (*CHEK2**1100delC) were at approximately threefold greater risk of breast cancer than noncarriers (9). Although low-penetrant genes such as *CHEK2* may not become useful targets for clinical genetic testing because of their modest impact on risk, they play an important role in the pathogenesis of a significant subset of breast cancers by virtue of their greater prevalence.

Time

For all forms of breast cancer, age is an important risk factor. Even women with inherited predisposition by virtue of alterations in *BRCA1* or *BRCA2* are typically diagnosed with breast cancer

after at least 20 years of life following puberty. For women without such an inherited predisposition, most breast cancer occurs in the postmenopausal years. This is probably because breast cancer arises as a consequence of a series of stochastic mutational events that together impart the requisite cellular attributes of an invasive malignancy. At time of diagnosis, breast cancers exhibit a wide range of somatic genetic and epigenetic changes. Preliminary answers to the question of just how many mutations a typical breast cancer harbors are coming from the application of methods for unbiased global surveys of genetic changes in cancers. In one such effort, the sequence of 13,023 protein-coding genes from 11 human breast cancers and 11 colorectal cancers was determined and analyzed. This Herculean effort required that ≈ 3 million polymerase chain reaction (PCR) products be generated and sequenced, yielding sequence information for over 460 million nucleotides of tumor DNA. After removing changes that were due to artifact, attributable to germ-line polymorphisms, or biologically silent (i.e., without effect on amino acid sequence), and making statistical adjustment for the expected rate of background “passenger” mutations, the authors concluded that individual breast cancers harbored an average of 12 mutant cancer genes (10). Their observation that no single tumor analyzed in this series had more than six mutant cancer genes in common with any other tumor in the panel underscores the remarkable complexity and heterogeneity of breast cancer, especially when one considers that cancers also contain multiple chromosomal changes and epigenetic alterations that would not have been detected by a purely sequence-based approach.

Virus

A viral etiology for mammary cancer in mice has been well established, and many of the important host genes dysregulated by insertion of the murine mammary tumor virus (MMTV) identified. Less well established, but no less intriguing, is the possibility that a significant proportion of human breast cancer is linked to infection with a human mammary tumor virus (HTLV). A candidate pathogenic HTLV, with overall 90% to 95% sequence homology to MMTV, has been identified. Further, genetic sequence from the presumptive HTLV env gene has been detected in 30% to 40% of breast cancers from America and Western Europe, but not normal breast tissue (11). Whether this virus is truly an etiologic agent in some human breast cancer has yet to be firmly established. Furthermore, the host genes within the human genome that are potential targets of insertional mutagenesis by this retrovirus have yet to be identified. As discussed in subsequent sections of this chapter, some of the human homologues of murine genes activated by MMTV are indeed important players in human breast cancer.

Molecular Attributes of Breast Cancer

Epithelial malignancies exhibit a wide diversity of molecular aberrations, reflecting the multiple genomic alterations that accumulate during carcinogenesis. Consequently, the behavior of cancers, even those of a seemingly unifying diagnosis such as breast cancer, can

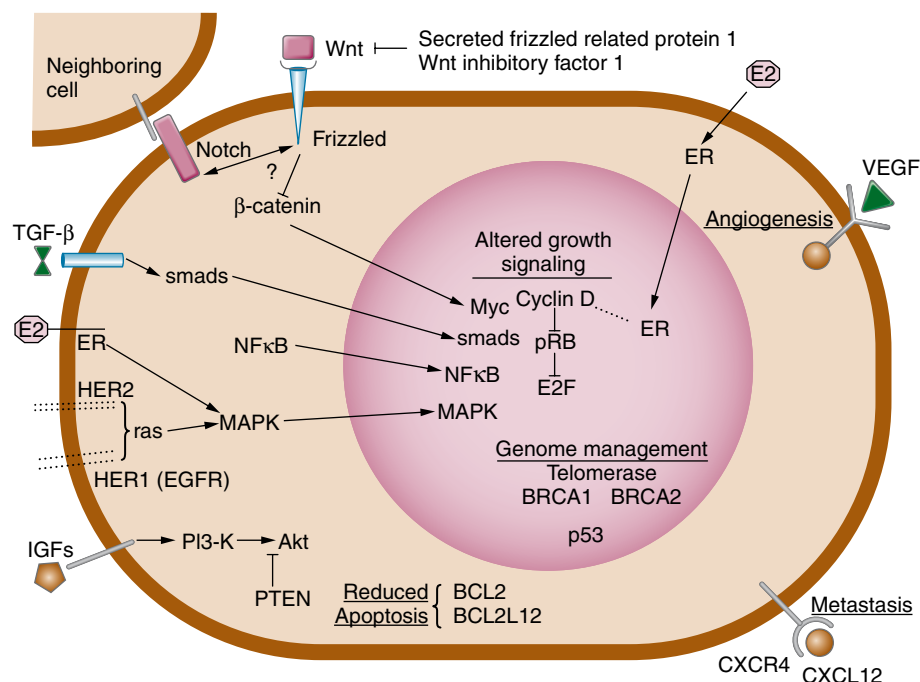


FIGURE 32-1 Cellular pathways involved in breast cancer development and progression. Portrayed on this schematic of a cell are the localizations and interactions among the molecular pathway disruptions that are discussed in this chapter as key elements of the molecular hallmarks of breast cancer.

be equally varied. That said, a common set of alterations in cell biology and tissue homeostasis are understood to constitute the required phenotypic changes that underlie the development and progression of cancer (12). The following sections consider and highlight the relevant molecular pathways in breast cancer that underlie these essential phenotypic traits of cancer. These pathways, and the relevant key molecules discussed in this chapter, are illustrated in Figure 32-1.

Autonomy of Growth Signaling

Oncogene overexpression via gene amplification is a common theme in human cancer, and one that is also evident in breast cancer. The most common chromosomal arms exhibiting significant gains in copy number in breast cancer are 1q, 8q, 16p, and 17q. One of the most commonly amplified genes in human breast cancer is the cyclin D1 gene, with reports of 13% to 21% of tumors with amplification of this genetic locus on 11q13 (13). Perhaps even more relevant to an appreciation of the role of cyclin D1 in breast cancer are observations that overexpression of the gene is evident in approximately half of human breast cancers, often without amplification of the 11q13 amplicon. Increased expression of cyclin D1 is seen in early stages of breast carcinogenesis, and primarily in association with ER-positive disease. Cyclin D1 is classically known as a mediator of hyperphosphorylation of the retinoblastoma protein (pRB) via its interaction with cyclin dependent kinases, such as cdk4. Hyperphosphorylation of pRB liberates E2F transcription factors, which, in turn, serve to stimulate cell proliferation. However, the mechanisms by which cyclin D1 promotes breast cancer are uncertain. There is evidence that cyclin D1 directly interacts with and affects the activity of the ER in a manner that is independent of its ability to bind cdk4 and thereby effect pRB phosphorylation. This interaction between ER and cyclin D may underlie the observed

association between cyclin D1 expression and the ER-positive phenotype in breast cancer. Indeed, by examination of gene expression profiles to identify signature patterns of gene expression following increased expression of wild-type versus mutant forms of cyclin D1 engineered to abrogate interaction with cdk, Lamb et al. have highlighted a cdk-independent impact of cyclin D1, not mediated through the activation of E2F, that may be of primary oncogenic relevance (13,14).

Another oncogene overexpressed via gene amplification in a significant proportion of human breast cancer is the transmembrane tyrosine kinase *HER2*, located on 17q. Amplification of *HER2* is evident in approximately 20% to 25% of invasive breast cancers and many high-grade cases of DCIS. *HER2* is a member of a family of interacting transmembrane tyrosine kinases that includes the EGF receptor, itself a target of gene amplification, albeit at a low frequency. Other than these somatic genetic changes in a notable subset of breast cancers, there does not appear to be a significant contribution of genetic mutation in protein kinases in human breast cancer. Indeed, a sequence-based analysis of the coding sequence of 518 protein kinases (involving approximately 1.3 Mb) in a panel of 15 primary breast cancers failed to reveal a previously unappreciated protein kinase mutational target in breast cancer (15). In contrast, most breast cancer cell lines exhibited mutations in various protein kinases.

Although the notion that chromosomal regions that display a high frequency of amplification in breast cancers harbor important oncogenes has facilitated the identification and understanding of some critical genes, such as *HER2*, this strategy has yet to bear fruit for a number of chromosomal regions. For instance, the search continues for the relevant oncogenes on the long arm of chromosome 1, which is one of the most commonly amplified chromosomal regions in breast cancers, both ER-positive and ER-negative (16). Similarly, the specific gene(s) that underlie the

relatively frequent observation of amplification of 16p are an active area of investigation (17).

The long arm of chromosome 8 is another region with frequent amplification. One potential proliferation-promoting oncogene that drives some, though no doubt not all cases of 8q amplification is *MYC*, located on 8q24. Estimates of *myc* amplification in breast cancer range between 5% and 40%, and associations with cancers of the medullary subtype and cancers arising in *BRCA1* mutation carriers have been reported (18,19).

In cancers that arise as a consequence of retroviral oncogenesis, certain host genes are converted into oncogenes by insertional mutagenesis. As discussed previously, in murine mammary carcinoma the MMTV promotes cancer by inappropriate activation of host genes. Two murine genes activated by MMTV in this manner, *Wnt* and *Notch*, have recently been implicated as oncogenes in human breast cancer. *Wnt* encodes a secreted protein that binds to a receptor on cells called Frizzled, resulting in inhibition of a complex that functions to phosphorylate β -catenin, thereby targeting it for degradation. Thus, *Wnt* signaling leads to cellular accumulation of β -catenin, which translocates into the nucleus and facilitates transcription of growth-promoting genes, including *c-myc* and *cyclin D1*. *Wnt* signaling is itself negatively regulated by secreted proteins that bind *Wnt* and prevent its interaction with Frizzled. Initial evidence that *Wnt* signaling was operative in human breast cancer came from observations that tumor specimens frequently exhibited elevated levels of β -catenin (20). More recently, decreased expression of two principle *Wnt*-binding negative regulators of *Wnt* signaling (secreted Frizzled-related protein 1 and *Wnt* inhibitory factor-1) have been reported in analyses of human breast cancer specimens. Interestingly, both of these genes were epigenetically silenced by CpG methylation in their respective promoter regions (21,22).

Another target of MMTV-mediated insertional mutagenesis in mice that has been implicated in human breast cancer is *Notch*. *Notch* genes encode transmembrane proteins that are key components in a signaling pathway initiated by its interaction with a membrane-bound protein ligand on the surfaces of a neighboring cell, an example of juxtacrine signaling (23). In recent years evidence of the relevance the *Notch* pathway in human breast cancer has arisen with reports of expression of *Notch*, *Notch* ligands, and downstream targets of *Notch* signaling (24,25). Intriguingly, studies aimed at elucidating the mechanism(s) by which *Wnt* promotes breast cancer implicated downstream effectors of *Notch* signaling, leading to experiments suggesting a complex interdependence between *Wnt* and *Notch* signaling in mammary oncogenesis (26).

Dampening of Antiproliferative Signaling

Genes that normally function to suppress or limit cell proliferation can, when lost, be involved in cancer progression. Indeed, loss of tumor-suppressor function is a common finding in carcinogenesis. For example, in cancers that arise in individuals who are heterozygous for mutations in tumor-suppressor genes such as *BRCA1*, *BRCA2*, and *p53*, the loss of (or blocked function of) the one remaining wild-type allele is a requisite early step in cancer

development. Although these genes have been implicated as exerting an antiproliferative effect, further studies have suggested that other consequences of their loss may be more determinative of their overall importance in breast cancer. Furthermore, simple loss of expression of tumor-suppressor genes is only one way in which they promote cancer. As an example, *p53* function can be altered by a variety of mechanisms and have a variety of consequences. It is understandable, therefore, that identification of a gene expression pattern reflective of *p53* status might be a more useful tool to evaluate the impact of *p53* (27).

One of the main antiproliferative signals in mammary epithelium is the transforming growth factor- β (TGF- β) pathway. Loss of responsiveness to TGF- β 's growth inhibitory effects is an important step in mammary carcinogenesis. However, as is the case for *p53*, the full story is complicated by the acquisition TGF- β -mediated responses that promote the malignant process. Many breast cancers produce elevated levels of TGF- β , which can function in both autocrine and paracrine fashions to enhance invasiveness and metastatic spread (28,29).

Reduced Apoptosis

In addition to a regulatory imbalance in favor of proliferation, many tumors have acquired measures to attenuate apoptosis. One of the most common mechanisms is a block of the pro-apoptotic function of *p53*, and breast cancers are among the many tumor types that display a high frequency of somatic mutation in *p53*. In addition to this, many breast cancers have been reported to display heightened activation of anti-apoptotic, so-called survival pathways. Among these is *BCL2*, which is expressed in approximately 65% of invasive breast cancers and imparts a favorable prognosis (30). More recently, a novel member of the *BCL2* family, *BCL2L12*, which is also expressed in approximately two thirds of human breast cancers, showed a statistically more significant association with longer disease-free and overall survival in a multivariate model incorporating lymph node status, age, grade, and hormone receptor expression (31). It is unclear whether the many reports of a favorable prognostic impact of *BCL2*, and *BCL2L12*, are related to the anti-apoptotic function of these proteins, presumably resulting in a greater measure of therapeutic responsiveness, or reflect some other important activity.

The phosphatidylinositol-3-kinase (PI3K) pathway is an important regulatory circuit mediating cell survival in mammary epithelial cells and breast tumors. This pathway begins with the interaction of various survival factors, such as the insulin-like growth factors, with their membrane-bound tyrosine kinase receptors, leading to phosphorylation of Akt, an important overall indicator of pathway activation. Of note, *PTEN*, the tumor-suppressor gene in Cowden syndrome, which includes an increased risk of breast cancer, is a negative regulator of this pathway. Immunohistochemical analyses of invasive breast cancers have reported that between 35% and 80% show phosphorylation of Akt, depending on the antibody used and phosphorylation site examined. Phosphorylation of the serum and glucocorticoid-induced kinase-1, another downstream effector of the PI3K survival pathway, was seen in about half of breast cancers (32). Interestingly, phosphorylation of Ser 473 of

Akt in a panel of metastatic breast cancers was shown to be predictive of resistance to endocrine therapy, suggesting the targeting this survival pathway might serve to promote the effectiveness of hormone-based therapies (33).

Another anti-apoptotic pathway that appears to play a role in a subset of breast cancer is the NF- κ B pathway. Activated NF- κ B enters the nucleus and binds to specific DNA sequences, affecting gene regulation. Using a DNA-binding assay, Biswas et al. examined extracts from human breast cancers and reported an association with the ER-negative subset. Supershift assays documented that the p50/p65 subunits were in this complex, and immunohistochemical analysis of tissue sections confirmed nuclear localization of p65, and thus NF- κ B activation, in the cancer cells themselves (34). Whether inactivation of NF- κ B will have therapeutic efficacy in ER-negative breast cancer, such as these authors showed in cell culture, remains to be seen.

Angiogenesis

The ability of tumors to generate an angiogenic response on the part of host cells, crucial for the delivery of oxygen and removal of metabolic waste, has been a well-recognized hallmark of cancers in general, and breast cancer in particular. One of the principle mediators of this activity is vascular endothelial growth factor (VEGF). Indeed, increased expression of VEGF and the physiologic correlate of increased microvessel density are evident at the noninvasive DCIS stage, and correlates with increased risk of an associated invasive cancer (35). VEGF levels can increase in response to estradiol and HER2-related signaling and can be released from the stroma by the action of matrix metalloproteinases. Strategies to counteract VEGF that are under study in the clinic include anti-VEGF antibodies, VEGF receptor inhibitors, and matrix metalloproteinase inhibitors. Hopefully, well-designed clinical trials, combined with translational studies, will further the development of anti-angiogenesis therapy as a valuable component to the management of breast cancer.

Invasion and Metastasis

It is commonly said that most patients with cancer die from metastatic disease. For breast cancer this can be extended: Were it not for the ability to metastasize, very few people would die from breast cancer. Being a cancer that arises in a readily accessible and nonvital organ, were it not for its propensity to metastasize systemically, breast cancer would today be a curable disease in nearly all women. Beyond the capabilities of unrestrained proliferation and diminished apoptotic responses essential for primary tumor growth, the formation of thriving metastatic deposits requires a complex array of cellular traits. These include the ability to disrupt normal tissue barriers, such as basement membrane, enter into and survive passage within a circulatory system of the body, and colonize a distant tissue site. As such, normal cells are unavoidably involved in this process. Indeed, as in angiogenesis, many of the steps in metastasis can be viewed as cancerous perversions of normal mechanisms by which epithelial cells interact with the stroma. Two excellent reviews relevant to this topic have been published;

a detailed recounting of the possible mediators of each step with the metastatic cascade in breast cancer is not possible within the confines of this chapter (36,37).

One of the more intriguing aspects of cancer metastasis, other than the simple fact that it happens so frequently, is the predilection of certain cancers for specific target tissue sites. This phenomenon is especially true for breast cancer. Indeed, the challenge of understanding the characteristic distribution of metastatic breast cancer attracted the attention of Paget over 100 years ago when he formulated his notion of “seed” and “soil” (i.e., tumor and metastatic site) as both playing important roles (38). A significant advancement in our understanding of the molecular features that underlie this seed-and-soil model came from analysis of the role of chemokines in this process, in analogy to their role in directing leukocyte trafficking. Many breast cancers express certain chemokine receptors, such as CXCR4, and in turn are stimulated when coming into contact with the chemokine ligands, such as CXCL12, that are produced by normal cells in the sites of metastatic spread. In model systems, blocking the interaction between CXCR4 on a breast cancer cell and its ligand, CXCL12, produced by lung tissue exerted a strong anti-metastatic effect (39).

Unlimited Replication

The capacity for limitless replication is directly correlated with expression of telomerase. Not surprisingly, nearly all invasive breast cancers have readily detectable telomerase activity. Of note, most, but not all, DCIS lesions also express telomerase, at similar levels (40). Whether the ability to maintain telomere length is a relevant determinant of which DCIS lesions will progress on to invasive breast cancer is as yet uncertain. Closely linked to the capacity for limitless proliferation mediated by expression of telomerase is the finding of breast cancer stem cells that are capable of self-renewal and differentiation. These cells, which constitute only a fraction of the total tumor mass, may well be the ultimate target, or victor, in the battle against breast cancer (41).

Future Directions

The application of new technologies for molecular analysis of breast cancer has given way to new insights into the nature of breast cancer, including pathogenesis, taxonomy, and therapeutic strategies. Chief among these new approaches has been the application of unbiased global gene expression analysis of patient specimens. In years prior, breast cancers were subcategorized according to pathologic type (e.g., ductal, lobular, medullary, etc.), histologic grade (also based on examination of tissue at the level of the light microscope), and expression of the hormone receptors ER α and PR. With the advent of gene expression profiling, human breast cancer can now be viewed as a collection of approximately five distinct subtypes: two with luminal cell features (referred to as the luminal A and luminal B groups), a HER2-overexpressing group, a subgroup characterized by the absence of hormone receptors and expressing many markers of basal epithelial cells (the

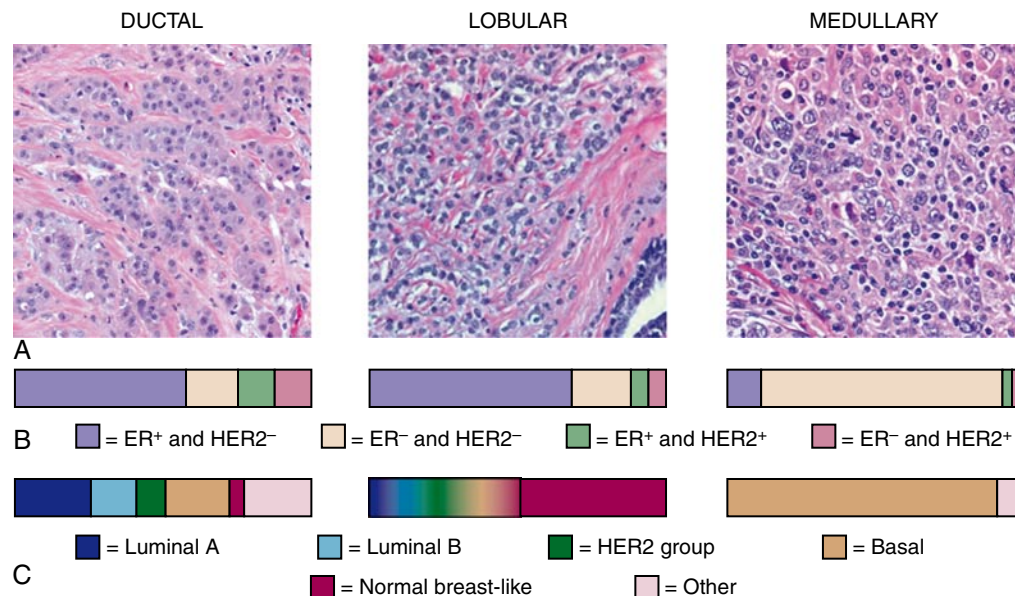


FIGURE 32-2 COMPARISONS BETWEEN CLASSIFICATION APPROACHES. **A:** Representative images of invasive breast cancers of three histologies (ductal, lobular, and medullary). (Courtesy of Dr. Wendy Wells, Department of pathology, Dartmouth-Hitchcock medical center, Lebanon, NH.) **B:** Relative proportions of cancers with the designated expression of estrogen receptor (ER) and amplification of HER2 for these three histologic types. **C:** An approximate breakdown of cancers when characterization is by microarray-based analysis of gene expression.

so-called basal phenotype group), and a minor subgroup that most resembles normal breast specimens in gene expression (42,43). It has been clear from these initial publications that classification of breast cancers according to patterns of gene expression corresponded remarkably well with clinically important subgroups, with varying prognoses and distinct therapeutic options. As this approach progresses, the taxonomy of breast cancer is slowing being rewritten. For instance, gene expression analysis comparing invasive ductal with invasive lobular breast cancers, which were poorly represented in the initial studies, suggests that the invasive lobular group of breast cancer should be subdivided into two fairly equally sized groups: One that clusters together and displays a pattern of gene expression that most closely resembles the normal breast-like subgroup and a second that is more heterogeneous and clusters with the typical invasive ductal subgroups (44). By a similar approach, gene expression profiling of medullary breast cancer, a somewhat uncommon pathologic type of breast cancer with characteristic histologic features, has shown that these cancers cluster with the basal phenotype subgroup (45). **Figure 32-2**

represents a comparison of ductal, lobular, and medullary breast cancers by three classification schemes, highlighting the underlying heterogeneity of this disease, and underscoring that these three histologic subtypes of breast cancer have common and distinctive phenotypes.

Gene expression profiling is now being applied as a prognostic indicator, to identify groups of patients who require systemic therapy to combat metastatic disease, be it overt or occult. Of equal importance, expression profiling holds promise as a valuable predictive tool of therapeutic efficacy for targeted therapies. Indeed, the examples of ER and HER2 in breast cancer are two instructive early examples of targeted therapy driven by analysis of gene expression, albeit each based on expression of a single gene. Looking ahead, novel strategies incorporating sophisticated molecular analyses of tumors into the implementation of clinical trials will be necessary to determine whether this approach can be expanded to identify prospectively subgroups of patients whose tumors depend on specific pathways or biologic processes that can be selectively targeted.

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Molecular Basis of Prostate Cancer

Prostate cancer is the most common noncutaneous malignancy and the second leading cause of cancer death in men in the United States (1). Prostate-specific antigen (PSA) screening began in the late 1980s and dramatically increased the diagnosis of this disease. An almost-simultaneous decrease in disease specific mortality has been noted (2). Whether this is a result of early and enhanced screening, early treatment of localized disease, early and aggressive treatment of micrometastatic disease, or other unknown reasons is a matter of considerable debate. Screening remains widespread but controversial due to lack of conclusive evidence demonstrating overall survival benefit. Prostate cancer has a very heterogeneous natural history and screening has resulted in overdiagnosis and overtreatment of men with indolent prostate cancer (3). Prostate cancer is not, as yet, curable once it has metastasized; however, differentiation of early-stage disease that ultimately will progress from disease destined to remain indolent is a major research priority. The molecular genetics of prostate cancer hold promise for the development of new screening and diagnostic tests to resolve this issue.

Several risk factors have been associated with prostate cancer including age, race, family history, and diet. Subsequently, tumor suppressors, oncogenes, and polymorphisms have been analyzed to help explain these risk factors. Epigenetic mechanisms, including DNA methylation and histone modifications, are important means of gene regulation and play essential roles in diverse biologic and disease processes. Common epigenetic mechanisms have been observed in prostate cancer affecting the expression and function of a large array of genes involved in tumorigenesis, tumor progression, and metastasis. The search for new and more specific biomarkers of disease continues with increased emphasis on epigenetic and genomic alterations predictive of metastasis and aggressiveness in this heterogeneous malignancy.

Pathology

Gleason Grade

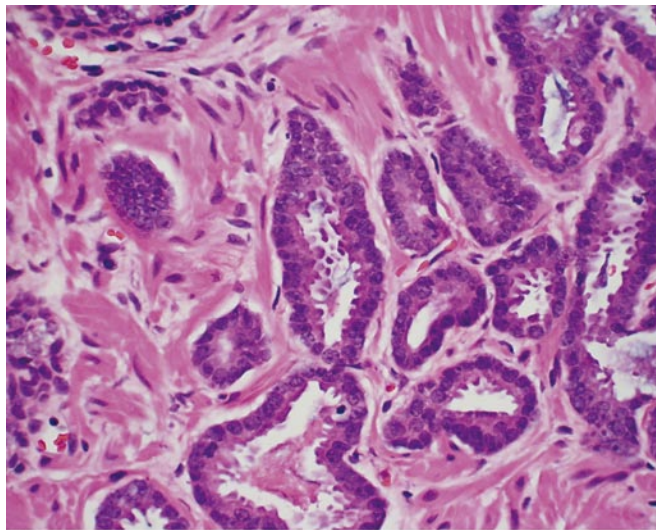
The Gleason grading system is the most commonly used pathologic grading system for adenocarcinoma of the prostate (4). Tissue samples are examined under low magnification, and the two most

common gland architectural patterns are assigned a grade from 1 to 5 and reported as a Gleason score. Figure 33-1 demonstrates the three most common Gleason scores. Gleason 1 and 2 are rarely seen in contemporary series of patients. Pathologic Gleason grade is the most important prognostic variable to the clinical risk assessment of newly diagnosed prostate cancer followed by tumor volume (5–9). The importance of Gleason pattern 4 and 5 volume has been correlated with subsequent pathologic stage, metastasis, and outcome (6).

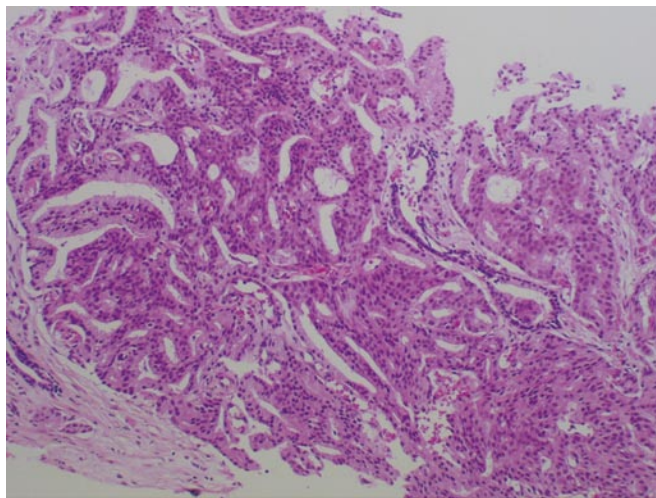
Prostate cancer is often multifocal, meaning that usually, multiple, distinct areas of malignancy exist within the prostate gland (10). The largest focus of disease is often called the index tumor, and the size of this tumor has been used in disease prognostication and prediction of metastasis (8). Qian and colleagues, in an analysis of genetic alterations in tumor foci and metastases, found that metastases were usually homologous with at least one tumor focus, but it was not always the index tumor (11). Several studies have described genetic heterogeneity within dominant tumor nodules and showed chromosomal differences between various areas of the same disease focus. Because of the genetic variability of prostate cancer and its multifocal nature, debate continues regarding the importance of smaller tumors and their impact on tumor progression and patient survival.

High-Grade Prostatic Intraepithelial Neoplasia

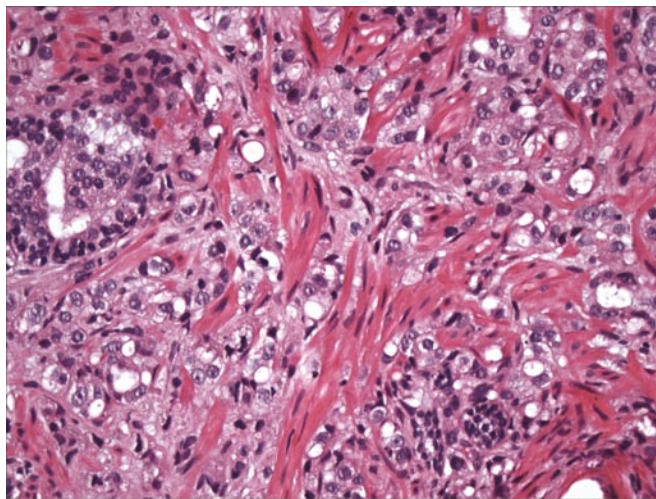
Often considered to be a precursor to adenocarcinoma of the prostate, high-grade prostatic intraepithelial neoplasia (HGPIN) consists of benign prostatic ducts lined with atypical epithelial cells. The incidence of HGPIN on biopsy averages 5.2% with a range from 1% to 25%, which is usually attributed to inter observer variability. The mean risk of prostate cancer diagnosis on subsequent biopsy in men with HGPIN is 26.4% (12). This risk is decreased in men who undergo extended-pattern biopsy schemes. Furthermore, HGPIN has been associated with progression to intermediate and high-grade adenocarcinomas, but has shown little association with low-grade disease. The most common chromosomal changes seen in HGPIN are losses at 8p and 13q (13). HGPIN, therefore, appears to be an intermediate step between benign and malignant disease in the molecular spectrum of prostate cancer.



A



B



C

FIGURE 33-1 EXAMPLES OF GLEASON GRADE 3, 4, AND 5 PROSTATE CANCER. GLEASON GRADE 3 SHOWS WELL-FORMED, SEPARATE GLANDS. **A:** Gleason grade 4 shows merging or cribriform glands. **B:** Gleason grade 5 is the most poorly differentiated and cancer cells no longer form glands but are visible as sheets of cells.

Molecular Pathology

Oncogenes and Tumor Suppressors

Oncogenes and tumor suppressors are otherwise normal genes whose expression has been changed or altered by mutation, deletion, amplification, or rearrangement, such that they contribute to the development of cancer. An oncogene is a gene that, when overexpressed, is associated with cancer. When loss of function of a gene due to mutation or deletion results in cancer, it is termed a "tumor-suppressor gene." Any given cancer is the end result of the combination of multiple oncogenes and tumor-suppressor genes. Oncogenes and tumor suppressors are associated with all human cancers. In prostate cancer, increased expression of many oncogenes and decreased expression of many tumor suppressors have been reported (14). Importantly, many of these genes are components of the same pathway, and multiple methods of genomic alteration may be responsible for altered gene function including polymorphism, mutation, deletion, translocation, methylation, and histone modification. Epigenetic alterations generally are not thought to cause cancer whereas genomic alterations are. Both genetic and epigenetic changes modify cancer progression (Table 33-1).

Hereditary Prostate Cancer

Family history is one of the strongest risk factors for the development of prostate cancer with a two- to eightfold higher risk of prostate cancer in men with an affected first-degree relative (15). Hereditary prostate cancer, however, is associated with familial clustering and high incidence of cancer among multiple first-degree relatives with a diagnosis before age 60. Approximately 9% of all cases are attributable to hereditary prostate cancer following an autosomal dominant susceptibility pattern. Prostate cancer susceptibility genes have been identified using lineage analysis of affected families. Significant linkage between chromosome 1q24–25, the HPC1 locus, and hereditary prostate cancer has been established (16). *RNASEL*, which lies within the HPC1 locus, encodes an endoribonuclease that mediates the activities of an interferon-inducible RNA degradation pathway. Polymorphisms of the *RNASEL* gene have been associated with increased prostate cancer risk. However, not all studies have confirmed these findings. Mutations in the ribonuclease L gene do not occur at a greater frequency in patients with familial prostate cancer compared with patients with sporadic prostate cancer (17). Genetic variants caused by polymorphisms or mutations in other genes, such as *PALB2*, *BRCA2*, the androgen receptor, 5- α -reductase type II, and *CYP17*, have been implicated in the development of hereditary prostate cancer.

Chromosomal Loss, Amplification, and Translocation

MSR1, *NKX3.1*, and *N33* are candidate tumor-suppressor genes that lie within the most commonly deleted regions on chromosome

Table 33-1 Oncogenes and Tumor Suppressors Implicated in Prostate Cancer

Gene	Chromosome/Locus	Function	
<i>UROC28</i>	6q23–24		Oncogene
<i>DD3</i> prostate-specific gene	9q21–22		Oncogene
<i>EZH2</i>	7q35	Gene silencing by histone modification	Oncogene
<i>NKX3.1</i>	8p21	Homeobox gene, regulates epithelial growth and differentiation	Tumor suppressor
<i>PTEN</i>	10q23	Lipid phosphatase	Tumor suppressor
<i>CDKN1B</i>	12p11–13	P27 cell cycle inhibitor	Tumor suppressor
<i>KLF6</i>	10p15	Zinc finger transcription factor	Tumor suppressor
<i>ERG/ETV1</i> (<i>ETS</i> family <i>TMPRSS2:ERG</i>)	21q22.3/7p21.2	Androgen-responsive fusion protein	Fusion oncogene
Retinoblastoma	13q14.1–14.2	Suppress cell division	Tumor suppressor
p53	17p13	Cell cycle control	Tumor suppressor
<i>CSMD1</i>	8p23 loss	CUB and Sushi multiple domains 1	Tumor suppressor
<i>MAP4K2</i>	11q13.1 gain	Mitogen-activated protein kinase 2	Oncogene
<i>MEN1</i>	11q13.1 gain	Multiple endocrine neoplasia	Oncogene
<i>SF1</i>	11q13.1 gain	Splicing factor 1	Oncogene
<i>PPP2R5B</i>	11q13.1 gain	Protein phosphatase2, regulatory subunit B isoform	Oncogene
<i>NAALADASEL</i>	11q13.1 gain	N-acetylated α -linked acidic dipeptidase-like	Oncogene
<i>EHD1</i>	11q13.1 gain	EH-domain containing 1	Oncogene

8p in prostate cancer. *MSR1* encodes a receptor on the macrophage cell surface that induces binding of oxidized low-density lipoprotein and other polyanionic ligands. Mutations, polymorphisms, or loss of the *MSR1* gene may compromise global macrophage function thereby exposing organs, including the prostate, to oxidative stress and damage. Although this gene does not code for prostatic proteins directly, oxidative stress has been implicated in the initiation of prostate carcinogenesis (13).

Loss of 8p23 in the region of the CUB and Sushi multiple domains 1 gene (*CSMD1*) has been associated with advanced prostate cancer (18). The retinoblastoma gene is also a tumor-suppressor gene and lies within the 13q loci. It is deleted in early prostate cancer development in animal models, prostate cancer cell lines, and some human prostate cancer specimens. Retinoblastoma inactivation in prostate cancer is the result of loss of heterozygosity and mutation. The 10q locus is lost in up to 45% of prostate cancers examined, and *MXI1* and *PTEN* are two putative tumor suppressors in this region. Regions of chromosome amplification in advanced prostate cancer include 8q containing the *MYC* gene and Xq11–13 encoding the androgen receptor (13). Gene amplification at 11q13.1 has been associated with disease recurrence. There are several candidate genes in this location (Table 33-1) but only *MEN1* and *MAP4K2* correlate with disease progression (18).

An example of translocation in prostate cancer is the fusion product *TMPRSS2: ERG*, which is created when *TMPRSS2* located at 21q22.3 and *ERG* located at 21q22.2 translocate. This

results in the overexpression of *ETS* genes in prostate cancer (19). *TMPRSS2: ERG* has been identified in up to 50% of prostate cancer surgical specimens and has been correlated with metastasis and disease-specific mortality (20). Because of its specificity for aggressive prostate cancer, it is potentially a good candidate biomarker for progression and requires further study in this regard, especially in patients considering active surveillance in lieu of initial treatment.

Wnt Signaling and β -Catenin

The Wnt signaling pathway plays a key role in embryonic development and is essential for the maintenance of stem cells. Wnt is an extracellular protein that interacts with the membrane-bound frizzled receptor to initiate its biologic activity. Wnt signaling leads to stabilization of β -catenin resulting in its nuclear accumulation. Nuclear β -catenin converts the TCF/LEF DNA-binding protein complex from a transcriptional repressor into a transcriptional activator. Inappropriate activation of the Wnt pathway is observed in many cancers and is putatively associated with tumor development.

In mice, β -catenin stabilization induces prostate intraepithelial neoplasia (PIN)-like lesions that are similar to early stages of human prostate cancer (21). In human prostate cancers, high levels of nuclear β -catenin are detectable by immunohistochemistry, whereas their levels are undetectable in normal prostate tissue. High levels of β -catenin expression are associated with the more

aggressive prostate tumors (22). Together, these findings imply that inappropriate activation of the Wnt signaling pathway can induce prostate cancer and progression.

There are several mechanisms by which the Wnt pathway may be inappropriately activated in prostate cancer; DNA methylation plays a key role in several of these processes. The adenomatous polyposis coli (*APC*) gene is hypermethylated in prostate tumors relative to samples of benign prostatic hyperplasia (BPH; 64.1% vs. 8.7%). *APC* is a key component of the β -catenin degradation complex. Thus, methylation-dependent silencing of *APC* can lead to β -catenin accumulation and Wnt pathway activation.

E-cadherin, a cell membrane protein, interacts with β -catenin and sequesters it at the inside surface of the cellular membrane. However, E-cadherin expression is often lost in prostate cancers due to promoter hypermethylation (23). Thus, because E-cadherin is no longer present, β -catenin is released into the cytoplasmic and nuclear compartments leading to Wnt pathway activation (24). Finally, the secreted-frizzled related proteins (SFRPs) and Wnt inhibitory factor-1 (*Wif-1*) sequester Wnt and antagonize Wnt signaling. Therefore, loss of *SFRP*/*Wif-1* expression can lead to Wnt pathway activation. The genes encoding several of the *SFRPs* and *Wif-1* are epigenetically silenced by DNA methylation in colorectal, lung, bladder, and kidney cancers and lymphocytic leukemia (25). In prostate cancer, *Wif-1* expression is strongly suppressed. The *SFRP1* gene is also aberrantly hypermethylated in prostate tumors relative to BPH tissue, and is partially to completely methylated in several human prostate cancer cell lines. These findings suggest that silencing of *Wnt* antagonist genes may play a role in prostate cancer development.

Xenobiotic Metabolism

Polycyclic aromatic hydrocarbons (PAHs) are toxic and carcinogenic compounds that are ubiquitous in the environment and are prevalent in cigarette smoke, automobile exhaust, and in charcoal-cooked meats. PAH exposure induces the expression of two cytochrome P-450s: *CYP1A1* and *CYP1B1*, which initiate PAH metabolism by oxidizing it (26). Following oxidation, PAHs are further modified by glutathione conjugation, ultimately leading to their detoxification and elimination from the body. Studies in knock-out mice reveal that *CYP1A1* induction is advantageous because animals that lack it are acutely sensitive to PAH toxicity. In contrast, *CYP1B1* induction has an adverse effect because *Cyp1b1*^{-/-} animals are protected against PAH toxicity. Experiments analyzing human prostate cancers and cell lines reveal that some cancers are unable to induce *CYP1A1* because its gene is silenced by DNA hypermethylation. In contrast, *CYP1B1* is overexpressed in prostate cancers due to gene hypomethylation. Thus, in prostate cancers a gene that protects against PAH toxicity is suppressed and a gene that mediates PAH toxicity is overexpressed. The combined effect, possibly synergistic, likely results in increased sensitivity to PAH toxicity. To compound this, these effects are further enhanced by the silencing by DNA hypermethylation of two

glutathione-s-transferases genes, *GSTP1* and *GSTM1*, which detoxify PAHs (27). Thus, some prostate cancers are likely to exhibit acute sensitivity to adverse PAH effects. Indeed, several large epidemiologic studies demonstrate that smokers, a group that has high PAH exposure, have higher prostate cancer-associated mortality (28). Future epidemiologic studies that assess genes involved in PAH metabolism may provide insights into this intriguing observation.

Polycomb Group Transcriptional Repression

The Polycomb group (PcG) proteins are developmental regulators that silence chromatin through epigenetic mechanisms. Enhancer of Zeste 2 (*EZH2*) is a member of the PcG proteins that is overexpressed in prostate cancer and is highly associated with tumor aggressiveness (29). Other studies reveal that *EZH2* is overexpressed and associated with aggressiveness in cutaneous melanoma, endometrial cancer, bladder cancer, and breast cancer. In addition, other PcG proteins, *BMI 1* and *RING 1*, are also overexpressed in aggressive prostate cancers (30). The *EZH2* complex silences gene expression by modifying histones to generate an inaccessible, heterochromatic chromatin configuration. In addition, *EZH2* associates with DNA methyltransferases, which hypermethylate and inactivate target genes. It is unclear how *EZH2* is overexpressed in prostate cancer. It is also unclear how *EZH2* overexpression increases prostate cancer aggressiveness. However, considering its ability to silence gene expression through epigenetic mechanisms, it is likely that *EZH2*'s effects are mediated by the silencing of particular target genes. Because genes involved in *Wnt* signaling and xenobiotic metabolism are epigenetically silenced in prostate cancers, it will be interesting to see if there is a link between *EZH2* and these pathways.

Micro-RNA

Micro-RNAs (miRNAs) are a recently discovered class of nonprotein-coding RNAs with evidence for diverse functions in development, cell differentiation, metabolism, and many disease processes including cancer. Several hundred have already been identified in human cells. Micro-RNAs are transcribed from the genome as long primary micro-RNAs, ranging from hundreds to thousands of nucleotides in size. The primary miRNAs are processed by an RNase III endonuclease, Droscha, into about 70-nt stem-loops called precursor micro-RNAs (pre-miRNAs), which are then exported into the cytoplasm by Exportin-5. In the cytoplasm, pre-miRNAs are further processed by a second RNase III endonuclease, Dicer, into about 22-nt mature mi-RNA. Micro-RNAs have been found to regulate gene expression mainly through inhibition of protein translation and mRNA degradation of target genes with which miRNAs have imperfect sequence complementarity. Down-regulation of genes by mi-RNA is largely mediated by a family of proteins called Argonaute, which form the effector complex RISC (RNA-induced silencing complex). It is predicted that each miRNA has hundreds of target genes, and a third of human protein-coding genes are regulated by miRNAs (31). An increasing body of evidence suggests that

miRNAs are involved in the initiation and development of different types of cancer including prostate cancer. Such evidence includes altered miRNA expression profiles in cancer cells and tissues, inactivation of particular genes by miRNAs in cancer, and identification of miRNAs as oncogenes or tumor-suppressor genes.

Micro-RNA Expression Profiling in Prostate Cancer

Several high-throughput miRNA expression profiling studies have found altered miRNA expression in prostate cancer, suggesting involvement of miRNAs in this disease. Volinia et al. (32) performed a miRNA microarray study on 56 malignant and seven benign prostate samples and identified 39 overexpressed and six underexpressed miRNAs (out of 228) in a sample of prostate cancer. In a similar study, Mattie et al. (33) found a distinct miRNA expression pattern that differentiates between hormone-sensitive LNCaP and hormone-insensitive PC3 prostate cancer cell lines, as well as benign and malignant prostate biopsy specimens. In accordance with miRNA expression alterations, miRNAs are also affected by genomic structural aberrations. By correlating the genomic location of 283 miRNAs with copy number alterations in prostate cancer, Lamy et al. (34) found that more miRNAs are located at regions with copy number gains.

Involvement of Micro-RNA Machinery in Prostate Cancer

Micro-RNAs depend on multiple proteins for biogenesis and function. Aberrations in any of these proteins will affect miRNA-mediated gene regulation. Chiosea et al. (35) studied the expression of several genes involved in miRNA processing and function and found the expression of Dicer is up-regulated in a significant fraction of prostate cancer and is associated with aggressive cancer features. Some other components of the miRNA machinery (XPO5, EIF2C2, EIF2C1, HSPCA, MOV10, and TNRC6B) are also up-regulated (35). These findings are consistent with miRNA expression profiling as mentioned previously, which shows that more miRNAs are up-regulated than down-regulated in prostate cancer.

By studying individual miRNAs and their target genes in cancer, several miRNAs have been identified as having tumor-suppressor or oncogenic characteristics. Sylvestre et al. (36) reported that miR-20a, a member of the *mir-17–92* cluster, affects prostate cancer PC-3 cell apoptosis by modulating the translation of the *E2F2* and *E2F3*. These mRNAs are members of the E2F family of transcription factors, which are essential in the regulation of the cell cycle and apoptosis (36). They found that inhibition of miR-20a makes PC-3 cells more sensitive to drug-induced apoptosis, whereas overexpression of miR-20a resulted in increased cell survival, suggesting an oncogenic role for miR-20a in prostate cancer.

Epigenetic Effects

Genetic alterations, such as mutations and epigenetic changes, contribute to the malignant transformation and progression of

prostate cancer. One of the earliest identified hallmarks of epigenetic alterations is DNA methylation—the addition of a methyl group to the 5'-carbon of cytosine in CpG sequences. This process is catalyzed by three DNA methyltransferases (DNMTs): DNMT1, DNMT3a, and DNMT3b. Methylcytosine residues are often found in short stretches of CpG-rich regions called CpG islands. These CpG islands are found in the 5' region of approximately 60% of genes and are generally 0.5 to 2 kb in length (37). With the exception of certain imprinted genes and genes on the inactive X chromosomes of females, most CpG islands exist in an unmethylated state. DNA hypo- and hypermethylation can occur and have been implicated in prostate cancer by causing chromosomal instability and transcriptional gene silencing, respectively. Figure 33-2 shows the progression from benign prostate tissue to malignancy via several different epigenetic alterations.

Hypermethylation

DNA hypermethylation is one of the most common and best-characterized epigenetic abnormalities in prostate cancer. Genes including classic and putative tumor-suppressor genes as well as genes involved in a number of cellular pathways, such as hormonal responses, tumor-cell invasion/tumor architecture, cell cycle control, and DNA damage repair, have been demonstrated to be hypermethylated. For many of these genes, promoter hypermethylation is often the mechanism responsible for their functional loss in prostate cancer. Inappropriate silencing of these genes can contribute to cancer initiation, progression, invasion, and metastasis. Some commonly hypermethylated genes in prostate cancer are discussed in the following sections (Table 33-2).

Androgen Receptor

The androgen receptor (AR) mediates testosterone and dihydrotestosterone activity, which is essential for the development and maturation of the prostate gland and prostate cancer. Most prostate cancer is initially androgen dependent, but eventually becomes androgen independent after androgen-deprivation therapy. Androgen-independent prostate cancers are characterized by a heterogeneous loss of AR expression. Genetic alterations that alter the sensitivity of the receptor to androgen, such as AR gene mutation and more commonly, amplification without loss of AR expression, are thought to play key roles in the development of androgen-independent prostate cancer (38).

Estrogen Receptors

The prostate expresses two types of estrogen receptors (ERs): ER α and ER β . Lost or decreased expression of ER α and ER β has been documented in prostate cancer and is associated with unresponsiveness to hormonal therapy with estrogens (39). Several studies have found hypermethylation to be the primary mechanism for inactivation of ER expression in prostate cancer. Furthermore, the extent of hypermethylation has been associated with tumor progression.

EPIGENETIC ABERRATIONS PHENOTYPE CHANGES

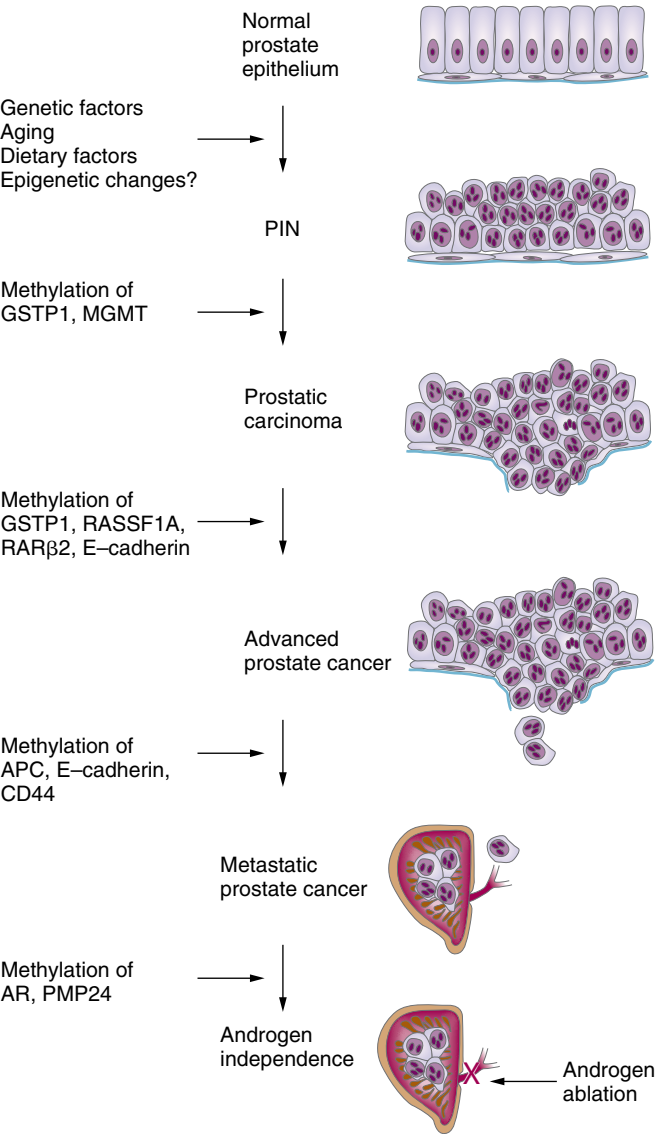


FIGURE 33-2 EPIGENETIC ALTERATIONS IN THE DEVELOPMENT OF PROSTATE CANCER. Multiple factors are associated with the development of prostate cancer including genetic predisposition, environmental factors, diet, ethnicity, and aging. Many of these factors modify the genome through epigenetic effects and DNA methylation may be an early event causing inactivation of DNA damage repair genes such as *GSTP1* and *MGMT*. Subsequent inactivation of cell-cycle control genes provides a growth advantage leading to locally advanced prostate cancer. Functional loss of genes in the cell adhesion pathway, such as *CD44*, may allow for metastasis. Ultimately, inactivation of AR via DNA hypermethylation allows cancer cells to become androgen insensitive. (From Li LC, Okino ST, Dahiya R. DNA methylation in prostate cancer. *Biochim Biophys Acta* 2004;1704:87–102, with permission.)

Cell Cycle Control Genes

An important characteristic of tumor cells is unbalanced proliferation due to impaired regulation of the cell cycle. The multiple checkpoints that control the cell cycle include the retinoblastoma protein, cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs). CDKIs are potential tumor-suppressor genes

Table 33-2 Epigenetic Changes in Prostate Cancer

Hypermethylation	Gene	Function
	Glutathione S-transferase P1 (<i>GSTP1</i>)	Detoxification if electrophilic compounds
	Ras association domain family protein isoform A (<i>RASSF1A</i>)	Unknown
	<i>AR</i>	Androgen effects +/-
	<i>ER</i>	Estrogen effects
	<i>CCND2</i> , <i>CDKN2A</i> , <i>CDKN1A</i> , <i>SFN</i>	Inhibit cyclin D-associated kinases, other cyclin dependent kinases
	<i>CD44</i> , <i>CDH1</i> , <i>LAMA3</i> , <i>LAMB3</i>	Cell architecture
	<i>MGMT</i>	DNA repair gene
	<i>DAB2IP</i> , <i>EDNRB</i> , <i>RASSF1</i>	Signal transduction
	<i>PTGS2</i>	Inflammatory response
Hypomethylation	<i>CAGE</i>	Novel testis antigen
	<i>HPSE</i>	Heparanase
	<i>PLAU</i>	Urokinase plasminogen activator
Histone modification	<i>VDR</i>	Vitamin D receptor
	<i>CPA3</i>	Carboxypeptidase A3
	<i>RARB</i>	Retinoic acid receptor β
	<i>PSA</i>	Prostate specific antigen
	<i>DAB2IP</i>	Tumor suppressor

that regulate cell cycle progression. Failure of cell cycle arrest due to alterations in CDKI expression has been implicated in prostate cancer. CDKIs are grouped into two families: the INK4 family and the CIP/KIP (kinase inhibitor protein) family. The INK4 family includes *CDKN2A* (p16), *CDKN2B* (p15), *CDKN2C* (p18), and *CDKN2D* (p19) and inhibits cyclin D-associated kinases (CDK4 and CDK6). The CIP/KIP family, which includes *CDKN1A* (p21), *CDKN1B* (p27), and *CDKN1C* (p57), inhibits most CDKs.

CDKN2A can be inactivated in prostate cancer by a variety of mechanisms including deletion, point mutation, and silencing by hypermethylation. Methylation-mediated inactivation of the *CDKN2A* gene has been reported in prostate cancer cell lines and prostate cancer tissue, with incidence rates ranging from 0% to 16% (40). Methylation at exon 2 of the *CDKN2A* gene is more frequent in prostate cancer tissue (66%) than methylation of the promoter region but exon 2 methylation does not

affect gene expression making the functional relevance of this epigenetic event unclear (42).

Tumor Invasion and Tumor Architecture Genes

The cadherin-catenin adhesion system is critical for the preservation of normal tissue architecture and is regulated by a family of proteins collectively termed “cell-adhesion molecules” (CAMs). Decreased expression of E-cadherin and other CAMs has been reported to have prognostic significance in various human cancers, including prostate cancer.

In prostate cancer, expression of E-cadherin is markedly suppressed and its promoter is methylated to varying degree. Kallakury et al. (42) reported an 80% prevalence of E-cadherin methylation in prostate cancer samples analyzed by methylation-specific polymerase chain reaction (PCR). In addition, methylation of the E-cadherin promoter is increased in advanced prostate cancer, making it a potential biomarker for cancer progression.

CD44

CD44 is an integral membrane protein involved in matrix adhesion and signal transduction. Loss of CD44 expression correlates with methylation of its gene promoter in prostate cancer and is associated with stage and prognosis (43). Other genes involved in the cadherin-catenin adhesion system have also shown methylation-mediated inactivation in prostate cancer such as H-cadherin, adenomatous polyposis coli (APC), caveolin-1 (CAV1), laminin α -3 (LAMA3), laminin β -3 (LAMB3), and laminin γ -2 (LAMC2).

DNA Damage-Repair Genes

DNA repair maintains genome stability during replication or following DNA damage. Cells defective in components of DNA repair pathways exhibit higher rates of spontaneous DNA mutations, which can lead to cancer. Hypermethylation of glutathione S-transferase Pi (GSTP1) and O6-methylguanine DNA methyltransferase (MGMT) have been reported in prostate cancer.

GSTP1

GSTP1, located at chromosome 11q13, belongs to a supergene family of glutathione S-transferases (GSTs) that play an important role in the detoxification of carcinogens and cytotoxic drugs by glutathione conjugation (44). GSTP1 inactivation may lead to increased cell vulnerability to oxidative DNA damage and the accumulation of DNA base adducts, which can precede other genetic alterations in carcinogenesis.

In prostate cancer, methylation of the GSTP1 gene promoter is the most frequently detected epigenetic alteration. Elevated CpG methylation has been detected in tissues with both atypia and PIN as well as in urine and ejaculate of men with prostate cancer (45). Because of its presence in body fluids at detectable levels, GSTP1 methylation is a potential epigenetic biomarker for prostate cancer.

Putative Tumor-Suppressor Genes

Functional loss of classic tumor-suppressor genes through DNA hypermethylation is a common event in prostate cancer. Some putative tumor-suppressor genes are silenced by DNA hypermethylation in prostate cancer, most notably, the Ras-association domain family-1 gene (RASSF1).

RASSF1

RASSF1 is located at 3p21.3 and encodes a protein similar to the RAS effector proteins. The biologic activity of RASSF1 is largely unknown although both in vitro and in vivo studies show that overexpression of RASSF1 in cancer cells leads to cell cycle arrest, reduced colony formation, and inhibition of tumor growth in nude mice. Thus, a tumor-suppressor role has been proposed for RASSF1. RASSF1 promoter methylation is a common event in prostate cancer and HGPIN, occurring in 54% to 96% and 64% of samples, respectively (46). Increased RASSF1 promoter methylation has been associated with high Gleason scores (40).

Other possible tumor-suppressor genes that are subject to epigenetic inactivation in prostate cancer include KAI1 (a prostate-specific tumor metastasis suppressor gene), inhibin- α (a member of the TGF- β family of growth and differentiation factors), and DAB2IP, a novel GTPase-activating protein for modulating the Ras-mediated signal pathway (47).

Hypomethylation

DNA methylation is a regulatory mechanism by which repetitive DNA is transcriptionally silenced to prevent it from propagating (48). Hypomethylation, or removal of methyl groups from normally methylated DNA, can disrupt this mechanism leading to structural and functional alterations of the genome.

Hypomethylation can be global, in which case there is an overall decrease in 5-methylcytosine content in the genome, or gene-specific, which refers to a decrease in cytosine methylation relative to the “normal” methylation level. Gene-specific methylation affects discrete regions of the genome, such as the promoter regions of proto-oncogenes or normally highly methylated sequences such as repetitive sequences and oncogenes. Both global and gene-specific hypomethylation have been implicated in human malignancy.

PLAU

The PLAU gene encodes urokinase plasminogen activator and is highly expressed in most prostate cancer tissues and invasive prostate cancer cell lines. Research evidence suggests that DNA methylation and gene amplification may participate in the regulation of the PLAU gene in prostate cancer. Hypomethylation of the PLAU promoter is associated with increased expression in hormone-independent prostate cancer cells, higher invasive capacity in vitro, and increased tumorigenesis in vivo (49). Other hypomethylated genes in prostate cancer include CAGE, a novel cancer/testis antigen gene, heparanase (HPSE),

CYP1B1, and *XIST*. *HPSE*, an endo- β -D-glucuronidase, and *CYP1B1* are overexpressed and substantially hypomethylated in prostate cancer compared with benign prostatic hyperplasia samples (50).

Histone Modification

DNA is organized into a nucleoprotein complex termed “chromatin.” The basic chromatin unit is the nucleosome, which is composed of 146bp of DNA wrapped around four pairs of histone proteins. The N-terminal tails of histones are positioned outside the nucleosome core and are thus susceptible to covalent modifications including acetylation and methylation. Acetylation and deacetylation of histone tails are catalyzed by histone acetyltransferases (HATs) and deacetylases (HDACs), respectively. Through histone acetylation, HATs have been shown to increase the activity of several transcription factors, including nuclear hormone receptors, which facilitate promoter access to the transcriptional machinery (51). Conversely, HDACs cause histone deacetylation, which is associated with transcriptional repression. Histone methylation is facilitated by histone methyltransferases (HMTs), which use S-adenosyl-methionine as a methyl donor group to the lysine and arginine residues of histone protein pairs H3 and H4. Like histone acetylation, histone methylation is reversible and is facilitated by at least two enzymes: lysine-specific demethylase1 (LSD1) and JmjC domain-containing histone demethylase1 (JHDM1).

EZH2

Accumulating evidence indicates that histone modifications play important roles in regulating gene expression during prostate cancer initiation, progression, and metastasis. Notably, the enhancer of the zeste homologue 2, *Drosophila* (*EZH2*) gene is involved in multiple epigenetic abnormalities. As mentioned previously, *EZH2* encodes a polycomb protein that contains a SET domain and thus has histone methyltransferase activity and can catalyze the addition of a methyl group to the histone at K27 (52). Varambally et al. (29) were the first to link the *EZH2* gene to prostate cancer by observing that *EZH2* is overexpressed in hormone-refractory and metastatic disease (29). Following this initial study, a number of important discoveries have been made indicating that *EZH2* may play a causal role in cancer progression. *EZH2* has been shown to silence the expression of *DAB2IP*, a putative tumor suppressor, in prostate cancer cells by adding methyl groups to H3-K27 on the *DAB2IP* promoter and inducing histone deacetylation (53). Furthermore, *EZH2* was found to control DNA methylation through direct physical contact with DNA methyltransferase (54). Thus, overexpression of *EZH2* in cancer could cause multiple epigenetic aberrations affecting a number of genes.

Prostate-Specific Antigen

Another gene that appears to be affected by histone methylation is the prostate-specific antigen (PSA) gene, which contains the androgen receptor response element in its 5' regulatory region.

Methylation of H3-K4 is associated with transcriptional inactivation of the PSA gene in the prostate cancer cell line LNCaP, and transcription of the PSA gene is accompanied by rapid decreases in di- and trimethylated H3 at lysine 4. In addition, a lysine-specific demethylase (LSD1) has been found to interact with the androgen receptor to stimulate the AR-dependent transcription of PSA in LNCaP cells by removing the methyl group at H3-K9. Metzger et al. (55) have identified an inhibitor of LSD1, pargyline, which can block AR-dependent transcription by blocking histone demethylation. The net effect of methylation on the PSA gene is not functionally significant, however, because PSA is a marker of disease progression rather than a causal factor.

Other Genes

Another important aspect of aberrant histone modification in prostate cancer is the loss of acetylation of H3 and H4 resulting from increased histone deacetylase (HDAC) activity. One study has directly demonstrated changes in histone acetylation associated with a particular gene locus by using the chromatin immunoprecipitation (ChIP) assay (47). Other indirect studies reported that treatment of prostate cancer cells with HDAC inhibitors increased expression of specific genes such as insulin-like growth factor binding protein-3 and carboxypeptidase A3 (CPA3), thereby suggesting a role for histone acetylation in aberrant gene regulation.

The 1,25-(OH)₂ vitamin D₃ acts through the vitamin D receptor to exert cell cycle regulatory antiproliferative effects in a variety of tumor cells, including prostate (56). Prostate cancer cells display a range of sensitivities to the antiproliferative effects of 1,25-(OH)₂ vitamin D₃, although the reasons for this range of sensitivity remain unclear. Prostate cancer cells that are insensitive to 1,25-(OH)₂ vitamin D₃ have increased levels of nuclear receptor corepressor SMRT (silencing mediator of retinoid and thyroid), which could result in increased deacetylase activity and decreased transcriptional activity of the vitamin D receptor. Combined treatment of prostate cancer cell lines with the HDAC inhibitor trichostatin A (TSA) and 1,25-(OH)₂ vitamin D₃ synergistically inhibits cell proliferation (57).

Future Directions

The search for new biomarkers is under way to aid in the diagnosis, prognosis, and decision-making process of men with prostate cancer. Due to the multitude of mechanisms of carcinogenesis, many approaches can be taken to accomplish these aims. Because epigenetic events are theoretically reversible, novel therapies that target hyper- or hypomethylated genes or histone acetylation implicated in prostate cancer aggressiveness and progression could bring new hope to patients with metastatic disease. Although the last two decades have been largely dedicated to the use and importance of PSA, the future of prostate cancer detection and treatment will be largely refined by discoveries in molecular biology.

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Molecular Pathogenesis of Epithelial Ovarian Cancer

Ovarian cancer is neither a common nor a rare disease. In 2006 in the United States, 20,180 women were diagnosed with ovarian cancer and 15,310 died from this malignancy (1). The lifetime risk for a woman to develop ovarian cancer is approximately 1 in 70. A small fraction of ovarian cancers can arise from germ cells or from granulosa theca cells, but more than 90% of ovarian cancers arise from epithelial cells. Although there has been some controversy regarding the origin of epithelial ovarian cancers, most are thought to develop from a single layer of flattened cells that cover the ovary or, more frequently, that line cysts immediately beneath the ovarian surface (2). Neoplasms with similar morphology and behavior can, however, arise from endometriosis, endosalpingiosis, fallopian tube, and the peritoneum.

Epithelial ovarian cancers exhibit a distinctive pattern of progression and metastasis (Figure 34-1). In its earliest stage, ovarian cancer cells invade the substance of the ovary and form a pelvic mass. Although epithelial ovarian cancers can spread hematogenously or through lymphatics, the most frequent route of metastasis is over the surface of the peritoneum. Indeed, if ovarian cancer arises from the surface of the ovary (or the fallopian tube), it can metastasize at a very early stage in the absence of a large localized mass. Multiple nodules of metastatic cancer can stud the peritoneal surface and form dense fibrous adhesions that bind adjacent loops of intestine producing mechanical obstruction (Figure 34-2). Retroperitoneal spread of cancer can invade the myenteric plexus producing paralytic ileus. Intestinal dysfunction through either mechanism produces symptoms of obstruction and prevents adequate nutrition. Ovarian cancer patients generally die from inanition or intercurrent infection. As local tumor control improves, distant metastases including brain metastases are likely to become of greater therapeutic concern.

Another distinctive feature of ovarian cancer is the formation of ascites fluid that contains leukocytes, peritoneal mesothelial cells, and a varying fraction of tumor cells. Accumulation of ascites fluid produces abdominal distention, which can be the initial symptom of disease. Fluid generally drains from the peritoneal cavity through diaphragmatic lymphatics, which can become occluded by tumor cells, preventing outflow (4). In addition, tumor angiogenesis produces incompetent vessels that permit greater efflux from the vascular compartment of protein-rich fluid into the peritoneal cavity. Ovarian cancer cells can produce copious amounts of vascular endothelial

growth factor/vascular permeability factor (VEGF/VPF) that is both an angiogenic factor and a permeability enhancing factor (5). Neutralization of VEGF with monoclonal antibodies blocks ascites formation in xenograft models (6) and in the clinical setting (7).

Given the location of the ovaries within the pelvic cavity and the difficulty in assessing abnormalities on routine gynecologic examination, the disease is diagnosed only after it has metastasized in approximately 80% of cases. Ovarian cancer is often described as a “silent killer,” but the disease is generally symptomatic, even at early stages in 89% of cases (8). Symptoms are not, however, specific and are generally attributed to benign gastrointestinal, genitourinary, musculoskeletal, or gynecologic conditions. Detection of a pelvic mass by physical examination or transvaginal sonography generally prompts exploratory surgery to remove the primary tumor and as much of the metastatic disease as possible, so called cytoreductive surgery. Chemotherapy is given for 4 to 6 months thereafter using a combination of cytotoxic drugs that often includes a platinum

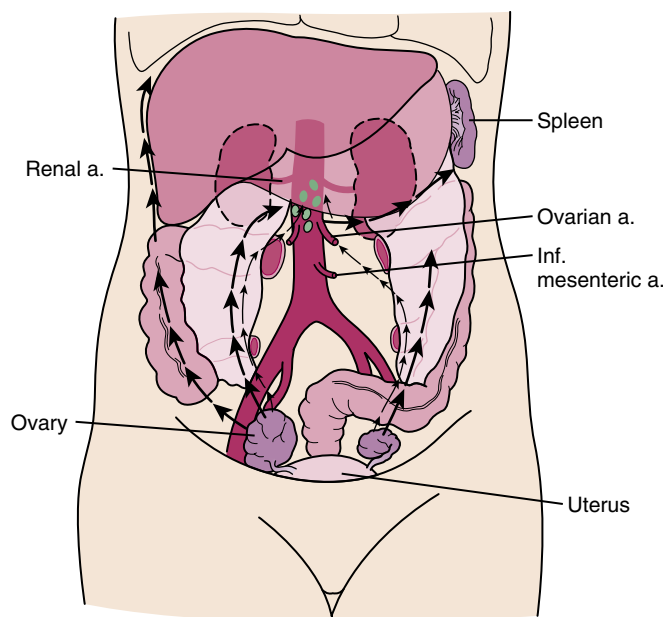


FIGURE 34-1 Intra-abdominal spread of ovarian cancer. (From Ref. 3, with permission.)



FIGURE 34-2 Intra-peritoneal metastases from epithelial ovarian cancer studding the peritoneal surface.

derivative and a taxane administered intravenously or into the peritoneal cavity.

In the 20% of patients with disease that is still localized to the ovaries (stage I), the prognosis is excellent, with up to 90% survival at 5 years using currently available surgery and chemotherapy. As the disease spreads to the other pelvic organs (stage II), to the peritoneal cavity and retroperitoneum (stage III) or to the hepatic parenchyma and beyond the diaphragm (stage IV) the prognosis becomes progressively worse, with a 5-year survival of less than 10% in the latter group. Five-year survival rates have improved significantly ($p < 0.05$) from 37% in the 1970s to 45% in the 1990s (1), related in large part to improvements in cytoreductive surgery and combination chemotherapy. Long-term survival for women

with advanced disease has, however, not improved dramatically over the last three decades and more than 70% of patients eventually succumb to the disease.

Cellular and Molecular Characteristics

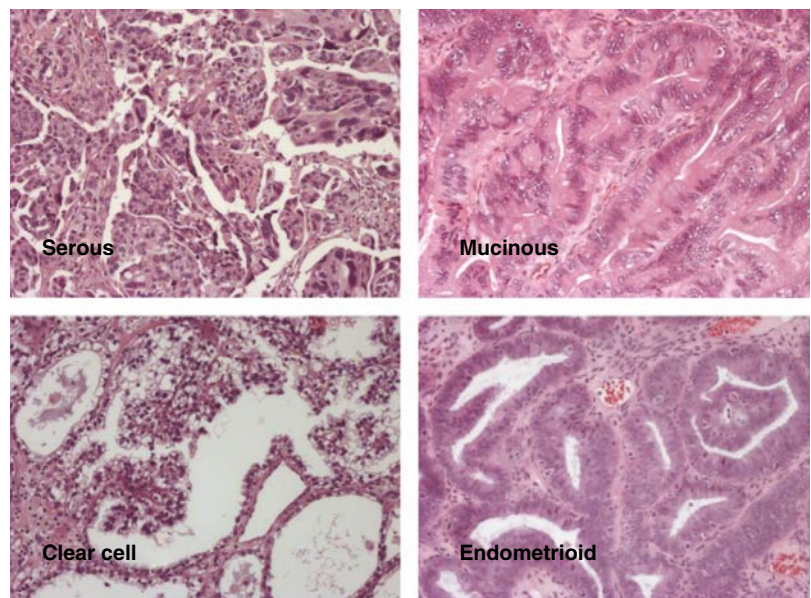
Heterogeneity of Ovarian Cancers

Epithelial ovarian cancers exhibit marked heterogeneity at a molecular, cellular, and clinical level. Differences in morphologic grade correlate with pathogenesis and prognosis. Low-grade cancers that are associated with a relatively better prognosis frequently express mutations of *Ras*, but not of *p53*; whereas most high-grade cancers have *p53* mutations, but *Ras* is rarely mutated. Borderline lesions of low malignant potential fail to invade the basement membrane, grow slowly, infrequently metastasize, and recur in less than 10% of cases when excised. In contrast to many invasive cancers, borderline tumors are most frequently diploid and exhibit few abnormalities on comparative genomic hybridization (CGH). Mutation of *Ras*, loss of heterozygosity (LOH) on Xq, microsatellite instability, and expression of amphiregulin occur more frequently in borderline lesions, whereas loss of estrogen receptor protein, LOH on 7q, LOH on 9p, and mutation of *p53* are detected more frequently in invasive cancers. Among cancers from different patients with invasive cancer, the fraction of cycling cells can vary from 1% to 79% with a mean of 9% to 34% in different series (9). Cyclin D1, cyclin E, and CDK2 are up-regulated in a minority of cancers with their DNA copy numbers or protein levels correlating inversely with survival. Conversely, the p16, p21, and p27 CDK inhibitors are down-regulated or mislocalized in a fraction of cancers, associated with a poorer outcome.

Ovarian cancers exhibit distinct histotypes—serous, endometrioid, clear cell, mucinous—that resemble epithelial components of normal fallopian tube, endometrium, vagina, endocervix, or intestine (Figure 34-3). Different histotypes differ with regard to epidemiology, genetic abnormalities, expression of tumor

FIGURE 34-3 Different histotypes of epithelial ovarian cancer.

HISTOTYPES OF OVARIAN EPITHELIAL CANCER



markers, and response to chemotherapy (10). Each histotype exhibits a distinctive pattern of gene expression judged by array analysis, real-time reverse transcriptase–polymerase chain reaction (RT-PCR), and immunohistochemistry (10). Alterations in ovarian cancers of different histotypes correlate with changes in the normal tissues that they resemble morphologically.

The HOX family of homeobox genes plays an important role in determining the histotype of ovarian cancers (11). During normal development, *HOXA9* is solely expressed in the primordia of the fallopian tubes, *HOXA10* in the developing uterus, *HOXA11* in the lower uterine segment and cervix, and *HOXA13* in the future upper vagina. Expression of these HOX genes is retained in adult tissues, but is not observed in ovarian surface epithelia. Expression of *HOXA9*, *HOXA10*, and *HOXA11* is recapitulated in serous, endometrioid, and mucinous epithelial ovarian cancers and enforced alterations in expression alter cellular histotype, indicating a causal role (11).

Immortalization

Telomerase activity is increased in 80% to 90% of ovarian cancers. Substantially greater telomerase activity is found in ovarian cancers than in borderline lesions or normal ovaries. Ovarian cancers demonstrate high degrees of genomic instability, possibly related to bridge-fusion breakage that occurs at telomeric crisis. Immortalization of human ovarian epithelial cells can be achieved by the introduction of human telomerase reverse transcriptase (hTERT) after disruption of the p53 and/or Rb pathways using SV40T/t antigen or siRNA against Rb (12). Cells immortalized with SV40 T/t and hTERT can be transformed with mutant Ras, producing cancers resembling human ovarian cancers that grow as nodules on the peritoneum and exhibit serous papillary histology (Figure 34-4; 13).

Clonality

As in many other malignancies, epithelial ovarian cancer is a clonal disease that arises from a single cell in more than 90% of cases (14). The clonality of sporadic invasive cancers that arise in the ovary

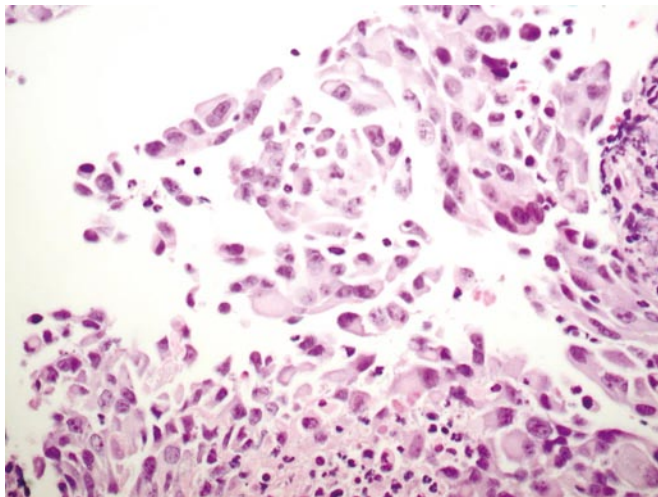


FIGURE 34-4 Histopathologic features of K-Ras-transformed, immortalized human ovarian surface epithelial cells. (From Ref. 12, with permission.)

contrasts with the multifocal origin of bilateral serous borderline cancers and of primary peritoneal cancers that arise sporadically or in carriers of germ-line *BRCA1* mutations. If most epithelial ovarian cancers are clonal diseases, multiple genetic changes must occur in the progeny of a single normal ovarian epithelial cell to produce malignant transformation. However, not all cells in the tumor must acquire the same additional mutations potentially contributing to intratumoral heterogeneity. Both germ-line and somatic alterations have been associated with epithelial ovarian cancers.

Genetic Abnormalities in Familial Ovarian Cancers

Approximately 90% of ovarian cancers are sporadic and not associated with a demonstrable pattern of inheritance. Of the 10% that are familial, most are associated with mutations of *BRCA1* and a smaller fraction with mutations of *BRCA2* or mismatch repair genes (human non-polyposis colon cancer syndrome; 15,16). Ovarian cancer can be associated with the Li-Fraumeni syndrome, but germ-line mutations of p53 are only rarely associated with ovarian cancer families. The lifetime risk of developing ovarian cancer depends upon the genetic defect: *BRCA1* (30%–60%), *BRCA2* (15%–30%), *HNPCC* (4%), and p53 (<1%). Interestingly, *BRCA1* and *BRCA2* are rarely mutated in sporadic ovarian cancers, but the genes can be silenced by methylation (17). Indeed, transcriptional profiles demonstrate similar changes in sporadic and *BRCA1*-associated tumors, suggesting that *BRCA1* function may be compromised in most ovarian cancers.

Genetic Abnormalities in Sporadic Ovarian Cancers

A number of genetic and epigenetic abnormalities have been detected in DNA from sporadic ovarian cancers including amplification, mutation, hypomethylation, deletion, loss of heterozygosity, and promoter methylation (Table 34-1; 18). Ovarian cancers are genetically unstable both in terms of DNA copy number and in exhibiting a mutator phenotype with greater change observed in high-grade cancers (Figure 34-5). For unknown reasons, areas of genomic aberration are associated with changes in RNA splicing. Aberrant expression of different splicing factors has been observed in ovarian cancer and altered splicing may not only affect adhesion (CD44), but also the activity of putative oncogenes (EVI-1b and AML-1), as well as the metabolism of xenobiotics (CYP1A1) or of multidrug resistance (SPF45). Abnormalities in miRNAs have been found in many different types of cancer related to gain or loss of DNA copies, methylation, or mutation (19). In ovarian cancers, copy number abnormalities have been found in 37% of 283 loci known to contain miRNAs. Some of the abnormalities were shared with breast cancers and/or melanomas, whereas others were unique to ovarian cancers.

Candidate oncogenes and tumor-suppressor genes have been mapped to most, but not all of the abnormal sites. Amplification of 1q22 is centered on the *RAB25* oncogene. Among the genes encoded on the 3q26 amplicon, protein kinase C iota (PKC ι), SnoN, ecotropic viral integration site-1 (EVI1), and the p110- α catalytic subunit of phosphoinositide-3-kinase (PIK3CA) are

Table 34-1 Genetic Abnormalities in Epithelial Ovarian Cancer

ACTIVATING EVENTS:	
AMPLIFICATION BY CGH:	1q22, 3q23-q27, 7q36, 8q24, 12p13, 20q13
MUTATION ² :	TP53 (62%), K-RAS (15%), BRAF (12%), CTNNB1 (12%), CDKN2A (10%), APC (9%), PIK3CA (8%), PTEN (8%), KIT (7%), MADH4 (7%)
HYPOMETHYLATION:	IGF-2, Sat2
INACTIVATING EVENTS	
DELETION BY CGH:	4p14, 4q22-q35, 5q, 6q22-q26, 7p15, 8p23-p21, 9q31-q33, 13q12-q34, 14q32, 15q14, 16p12-q24, 17p13-q21, 18q21-q33; 22q11-q13; Xp, Xq
LOH	(> 50%): 17p13, 17q21 (> 30%): 1p, 3p, 5q, 5q, 6q, 7q, 8q, 9p, 10q, 11p, 13q, 18q, 19p, 20; Xp
PROMOTER METHYLATION ³	ARHI, BRCA1, BRCA2, DAPK, H-CADHERIN, MLH1, ICAM-1, LOT-1, MCJ, MUC2, OPCML, PACE-4, PEG3, p16, p21, RASSF1, SOCS1, SOCS2, TMS/ASC, 14-3-3σ,
INHIBITION OF GROWTH BY CHROMOSOME TRANSFER	2, 3, 7 AND 22

CGH, comparative genomic hybridization; LOH, loss of heterozygosity.
1 Personal Communication, Dr. Joe Gray, Lawrence Berkeley National Laboratory, Berkeley, California, 2007.
2 www.Sanger.ac.uk
3 From Balch C, Huang T, Brown R, et al. The epigenetics of ovarian cancer drug resistance and resensitization. *Am J Ob Gyn* 2004;191:1552–1572, with permission.

overexpressed in a fraction of ovarian cancers and are associated with a poor prognosis. PKCιota protein is required for the establishment and maintenance of epithelial cell polarity. Levels of aberrant PKCιota are markedly increased and/or mislocalized in all serous

ovarian cancers and are associated with increased cyclin E protein expression and proliferation (19). *EVII* and the closely associated *MDS* gene have previously been implicated in acute myelogenous leukemia. *EVII* and *MDS1/EVII* gene products increase cell proliferation, migration, and decrease transforming growth factor-β-mediated plasminogen activator inhibitor-1 promoter activity in ovarian epithelial cells. Another amplicon on chromosome 19q contains the p85 β subunit of PI3K as well as AKT2, a target of PI3 kinase. The FGF-1 peptide growth factor encoded by a gene in the amplicon at 5q31 can stimulate cancer growth, stromal growth, and angiogenesis. An amplicon at 8q 24 contains c-myc, which is amplified in up to 40% of ovarian cancers, inducing factors required for proliferation and activating telomerase. Another major amplicon at 20q13.2 contains the *BTAK/Aurora* kinase gene that up-regulates c-Myc and activates telomerase.

Loss of Tumor-Suppressor Function

In ovarian cancers, loss of function has been observed in several putative tumor-suppressor genes (Table 34-2). In some cases, inactivating mutations have been associated with LOH (*BRCA1*, *BRCA2*, *PTEN*), but in others epigenetic changes alone (*RASSF1A*, *DLEC1*) or in combination with LOH (*ARHI*, *LOT-1*, *PEG3*, *WVVOX*) have silenced suppressor function. Although abnormalities can occur in *RB*, *WT*, and *VHL*, loss of their function is uncommon in epithelial tumors of the ovary.

With a few possible exceptions, such as the use of talc products, chemical carcinogens have not been linked to ovarian cancer. Elevated risk has associated with increased numbers of ovulatory cycles lifelong, including early menarche, late menopause, and nulliparity. Conversely, multiple pregnancies, prolonged breast-feeding, and the use of oral contraceptives decrease the risk of ovarian cancer in later life. This has led to the hypothesis that otherwise quiescent epithelial cells covering the

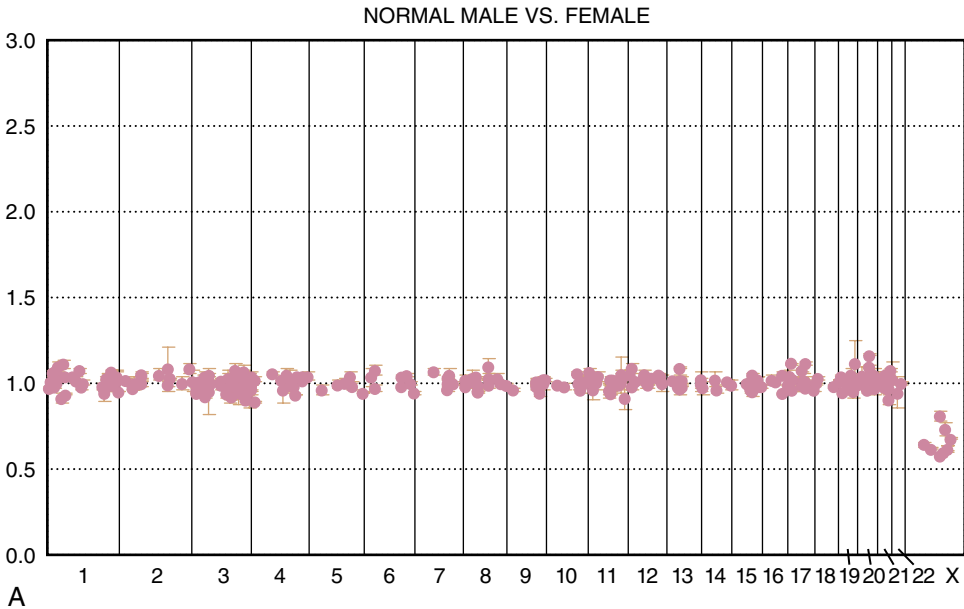


FIGURE 34-5 COMPARATIVE GENOMIC HYBRIDIZATION (CGH) ANALYSIS OF DNA FROM NORMAL CELLS AND OVARIAN CANCERS OF DIFFERENT GRADES. **A:** Normal male and female DNA with differences in the X and Y chromosomes.

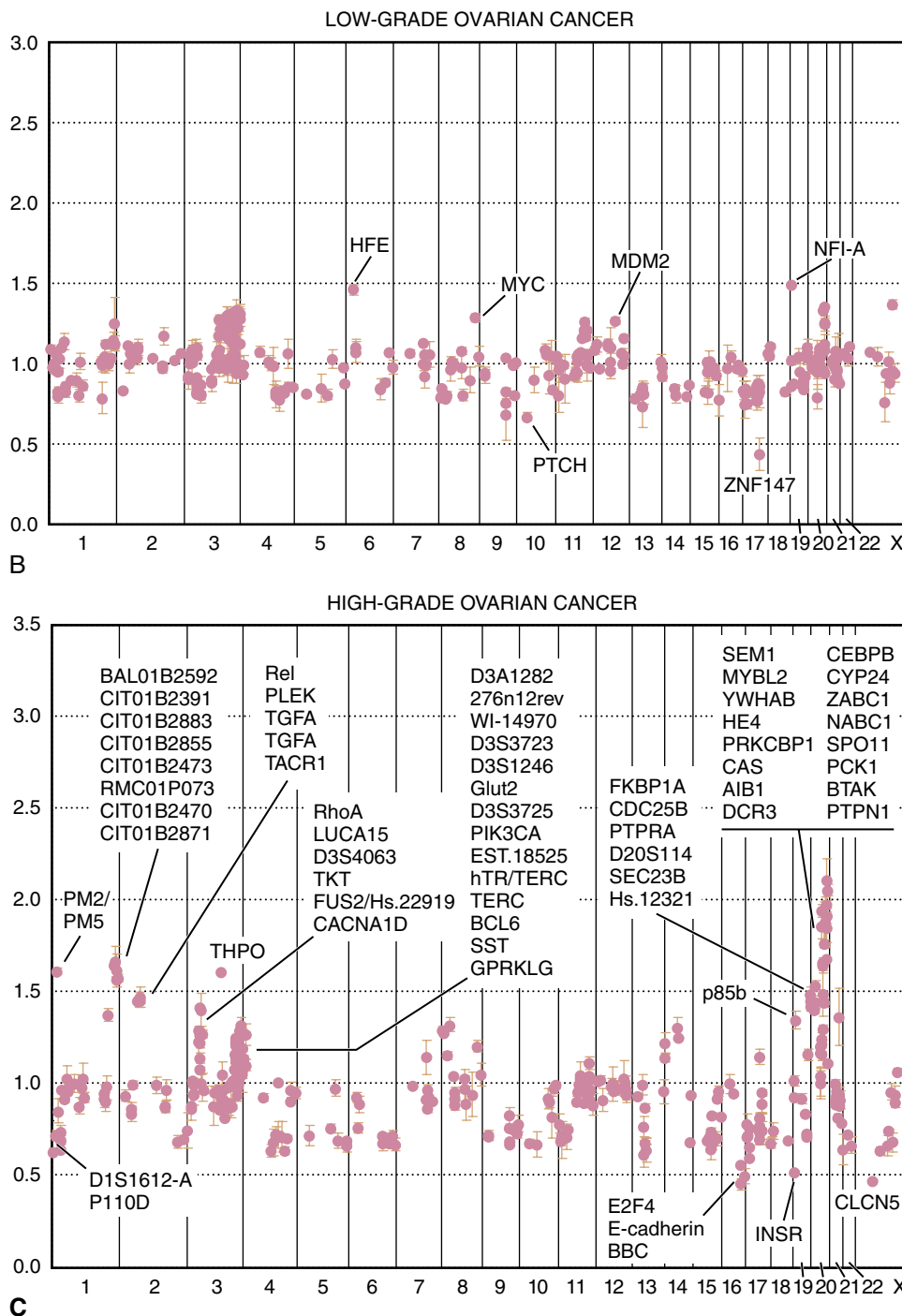


FIGURE 34-5—CONT'D B: Low-grade ovarian cancer with amplicons and deletions noted. C: High-grade ovarian cancer with substantially greater abnormalities in copy number.

ovary undergo “spontaneous” mutation during the proliferation required for repair of ovulatory defects. After menopause, further proliferation could occur in response to follicle-stimulating hormone (FSH), leutinizing hormone (LH), estrone, and androgen. The pattern of transitions, transversions, and deletions within mutated *p53* genes in ovarian cancers resembles the pattern of mutations in factor IX deficiency (hemophilia B) in the germ line that is thought related to spontaneous deamination during DNA replication (20). If spontaneous mutation during proliferation

is a critical mechanism driving carcinogenesis, genetic events requiring only a “single hit” may be favored.

Due to the dominant negative activity of mutant TP53 protein, *p53* function can be lost with a single genetic event. Frequent abnormalities have been detected in *p53*, where mutations occur in up to 70% of advanced-stage ovarian cancers. Consistent with the importance of ovulation in the mutation of *p53*, the frequency of *p53* mutations in ovarian cancers has been correlated with the number of ovulatory cycles previously experienced by patients in

Table 34-2 Putative Tumor-Suppressor Genes in Epithelial Ovarian Cancer

Gene	Chromosome	Down-Regulated or Inactivated	Mechanisms of Down-regulation	Function
<i>ARHI (DIRAS3)</i>	1p31	60%	Imprinting; LOH; Promoter Methylation; Transcription down-regulated by E2F1 and E2F4	26-kD GTPase; inhibits proliferation and motility; induces autophagy and dormancy; up-regulates p21; inhibits cyclin D1, Pl3K, Ras-MAP, Stat3
<i>RASSF1A</i>	3p21		Hypermethylation	Inhibits proliferation and tumorigenicity in many different cancers. Interacts with Ras inhibiting or down-regulating cyclin D and signaling through JNK, stabilizes microtubules, regulates spindle checkpoint and fas- and TNF-induced apoptosis.
<i>DLEC1</i>	3p22.3	73%	Promoter hypermethylation and histone hypo-acetylation	166-kD cytoplasmic protein that inhibits anchorage dependent growth
<i>SPARC</i>	5q31	70%–90% decreased expression; 9% lost	Transcription	32-kD Ca ⁺⁺ binding protein; prevents adhesion
<i>DAB-2 (DOC2)</i>	5q13	58%–85% lost	Transcription	105-kD protein binds GRB2 preventing Ras/MAP activation, prevents c-fos induction and decreases ILK activity, contributing to anoikis and inhibiting proliferation and anchorage-independent growth and tumorigenicity
<i>LOT-1 (ZAC1)</i>	6q25	39%	Imprinting; LOH; Transcription down-regulated by EGF, TPA	55-kD nuclear zinc finger protein inhibits proliferation and tumorigenicity
<i>RPS6KA2</i>	6q27	64%	Monoallelic expression in ovary; LOH	90-kD ribosomal S6 serine threonine kinase that inhibits growth, induces apoptosis, decreases pERK and cyclin D1, increases p21 and p27
<i>PTEN (MMAC-1)</i>	10q23	3%–8% mutated; expression lost in 27%, particularly in endometrioid and clear cell histotypes	Promoter methylation; LOH; mutation	Pl3-phosphatase; decreases proliferation, migration and survival; decreases cyclin D and increases p27
<i>OPCML</i>	11q25	56%–83%	Promoter methylation; LOH; mutation	GPI-anchored IgLON family member; induces aggregation; inhibits proliferation and tumorigenicity
<i>BRCA2</i>	13q12–13	3%–6%	Mutation; LOH	Binds RAD51 in repair of DNA double-stranded breaks (DSBs)
<i>ARLTS1</i>	13q14	62%	Promoter methylation	ADP ribosylation factor induces apoptosis
<i>WWOX</i>	16q23	30%–49%, particularly in mucinous and clear cell histotypes	LOH; mutation	Decreases anchorage independent growth and tumorigenicity; mouse homologue required for apoptosis
<i>P53</i>	17p13.1	50%–70%	Mutation	53-kD nuclear protein induces p21 with cell cycle arrest promoting DNA stability; induces apoptosis
<i>OVCA1</i>	17p13.3	37%	LOH	50-kD protein; decreases proliferation and clonogenicity; decreased cyclin D1
<i>BRCA1</i>	17q21	6%–8%	Mutation; LOH	E3 ubiquitin ligase that participates directly in repair of DNA DSBs through homologous recombination; Regulates c-Abl; induces p53, androgen receptor, estrogen receptor and c-myc
<i>PEG3</i>	19q13	75%	Imprinting; LOH; Promoter methylation; Transcription	Induces p53-dependent apoptosis

LOH, loss of heterozygosity.

some, but not all studies (21). Mutations of *p53* have generally not been detected in benign cystadenomas. Some 15% of frankly invasive cancers in stage I have *p53* mutations consistent with the hypothesis that mutation of *p53* is a relatively late change in tumor progression, correlating with acquisition of metastatic potential.

Function of imprinted growth regulatory genes can also be lost in a single genetic or epigenetic event. Approximately 70 human genes are imprinted with only one allele expressed at conception, during embryonic development, and in each normal adult cell. Silencing of the maternal or paternal allele is inherited epigenetically. Among the candidate tumor-suppressor genes whose function is down-regulated in ovarian cancer (Table 34-2), at least three are imprinted: *ARHI* (*DIRAS3*), *LOT-1*, and *PEG3*. *ARHI* encodes a 26-kD GTPase with homology to ras and rap that is down-regulated in more than 60% of ovarian cancers, associated with decreased time to progression (22). Expression can be down-regulated by multiple mechanisms including LOH, methylation and silencing of the functional allele, transcriptional regulation with E2F1 and E2F4, and shortened mRNA half-life. Re-expression of *ARHI* at physiologic levels inhibits clonogenic growth and motility of ovarian cancer cells that lack its expression, inhibiting STAT3 translocation, down-regulating cyclin D1 and inducing p21^{WAF1/CIP1} and p27^{KIP1}. *ARHI* re-expression decreases levels of *HIF1-α* and inhibits signaling through Ras/MAP and PI3K/TSC2/mammalian target of rapamycin (TOR), inducing autophagy (23). Among the other imprinted tumor suppressor genes, *LOT-1* is a zinc finger protein that presumably acts as a transcription factor (24) and *PEG3* is required for *p53*-induced apoptosis. Consequently, imprinted genes may regulate the proliferation, motility, and survival of ovarian epithelial cells through multiple mechanisms.

Activation of Oncogenes

Several known oncogenes are amplified, overexpressed, or mutated in ovarian cancers (Table 34-3). The two most common aberrations, mutations of members of the PI3K/AKT pathway and of the RAS/RAF pathway are discussed in the following sections. Abnormalities of receptor and nonreceptor kinases also have been documented.

Despite early reports that HER-2 was overexpressed and potentially amplified in 20% to 30% of invasive epithelial ovarian cancers, overexpression was found in only 11% of 837 ovarian cancers in a Gynecologic Oncology Group trial in which trastuzumab produced objective responses in only 7% of tumors with HER-2 overexpression (25). In contrast to observations in lung cancer, the tyrosine kinase domain of *EGFR* is rarely mutated in ovarian cancer, but the receptor can be amplified in up to 20% of cases. A characteristic constitutively activating deletion in the extracellular domain of *EGFR* (*EGFRvIII*), first demonstrated in glioblastomas, has also been detected in a small fraction of ovarian cancers (26). Constitutive activation of *EGFR* has, however, not been detected in ovarian cancer cell lines (27), consistent with the modest clinical activity of erlotinib and gefitinib in ovarian cancer where objective regression has been observed in 4% to 6% of cases.

BTAK/aurora A kinase, a serine-threonine kinase required for chromosome segregation and centrosome function, is amplified in 10% to 25% and activated in 48% of ovarian cancers (28). BTAK/aurora A kinase regulates telomerase activity. Forced expression of aurora A induces centrosome amplification, cell cycle progression, and chemoresistance to cisplatin and paclitaxel mediated through AKT in a *p53*-dependent manner. Chemical inhibition

Table 34-3 Oncogenes Associated with Epithelial Ovarian Cancer

Gene	Chromosome	Amplified	Overexpressed	Mutated	Function
- Rab25	1q22	54%	80%–89%		Cytoplasmic GTPase
- Evi -1	3q26	—	43%	8%–12%	Transcription factor
- eIF-5A2	3q26	44%	—	<1%	Elongation factor
- PKCi	3q26	9%–22%	78%	2%–24%	Cytoplasmic serine-threonine kinase
- p110 PI3K	3q26	—	32%	Rare	Cytoplasmic lipid kinase
- FGF-1	5q31	20%	51%		Growth factor for cancer and angiogenesis
- Myc	8q24	11%–20%	41%–66%		Transcription factor
- EGFR	7p12	20%–21%	9%–28%		Tyrosine kinase growth factor receptor
- Notch-3	9p13	5%	62%		Cell surface growth factor receptor
- K-Ras	12p11–12	6%–11%	30%–52%		Cytoplasmic GTPase
- HER-2	17q12–21	12%–36%	4%–12%		Tyrosine kinase growth factor receptor
- p85 PI3K	19q	12%–27%	42%–63%		Cytoplasmic lipid kinase
- Cyclin E	19q12	10%–15%	12%		Cyclin
- AKT2	19q13.2		48%		Cytoplasmic serine-threonine kinase
- BTAK/Aurora A	20q13				Nuclear serine-threonine kinase

of BTAK/aurora A kinase down-regulates NF- κ B, Bcl-XL, and Bcl-2 and enhances sensitivity to chemotherapy.

RAB25, a member of the RAB family of small G-proteins implicated in apical vesicle trafficking, is amplified and overexpressed in approximately half of ovarian cancers and is associated with a poor prognosis. Forced expression of RAB25 protein markedly increased anchorage-dependent and anchorage-independent proliferation, prevented apoptosis and anoikis, including that induced by chemotherapy, and enhanced growth in xenografts (29). Inhibition of apoptosis was associated with a decrease in the expression of the pro-apoptotic molecules BAK and BAX, as well as activation of the PI3K/AKT pathway.

In addition to the kinases, growth factors, and signal transducing proteins members of the family of eukaryotic initiation factors (eIFs) have been implicated in ovarian oncogenesis. The initiation factor eIF-5A2 maps to a region on 3q26 that is amplified in ovarian cancers. Overexpression of eIF-5A2 is associated with advanced-stage ovarian cancer (30). Forced expression of eIF-5A2 stimulates and antisense inhibits ovarian cancer growth and tumorigenicity. Several transcription factors are also overexpressed in ovarian cancers. The *c-Myc* gene is amplified in up to 40% of ovarian cancers and protein levels are increased in approximately one third of cases, including clear cell and endometrioid histotypes (31). The *c-MYC* protein induces E2F1, E2F2, E2F3, and telomerase, while blocking *p53*-mediated transcription of *p21*. Despite the probable importance of *c-Myc* amplification in oncogenesis, no apparent association with survival has been found.

To explore requirements for oncogenesis, ovarian epithelial cells from transgenic mice have been engineered to express different combinations of oncogenes. When target cells were derived from transgenic mice that lacked *p53* expression, the addition of any two of the three oncogenes—*c-myc*, activated K-ras, and activated Akt—were sufficient to induce ovarian tumor formation (32). In mice that were deficient for both *p53* and *Brca1*, *Myc* is sufficient to induce transformation, but *Myc* is not sufficient to induce transformation in mice deficient in either *p53* or *Brca1* (33). Consistent with the reported platinum chemosensitivity in patients with *BRCA1*-associated ovarian cancers, the *Brca1*-deficient murine ovarian cancers have increased sensitivity to the DNA-damaging agent cisplatin, whereas sensitivity to the microtubule poison paclitaxel is similar for *Brca1*—wild-type and *Brca1*-deficient mice. Cancers generated with different oncogenes differ in response to inhibitors of signaling pathways. The mTOR inhibitor rapamycin is active against cancers generated with *c-myc*/Akt or K-ras/Akt, but not against cancers generated with K-Ras/Myc on a *p53* null background (34).

Aberrant Signaling Pathways

Several type I tyrosine kinase growth factor receptors can be activated in epithelial ovarian cancers including EGFR, HER-2/HER-3, M-CSFR, IGFRI, and PDGFR. Binding of relevant ligands to one or more of these receptors can activate signaling through RAS/MAP, PI3K, JNK, JAK/STAT, PKC, and PLC γ or β with a concomitant alteration in calcium fluxes. Among these several signaling pathways, the RAS/MAP and PI3K pathways

appear to be particularly important in regulating the growth, survival, metastatic potential, and drug resistance of ovarian cancer cells.

In many tumor types, K- or H-ras is activated by mutations at codons 12 or 61, permitting constitutive binding of GTP rather than GDP to the RAS protein. Activated Ras can signal through the MAPK, PI3K, or Ral pathways affecting proliferation, survival, motility, invasion, and drug resistance. The *Ras* gene is mutated in less than 20% of invasive serous ovarian cancers (35), although it can be mutated in as many as 50% to 80% of the less common mucinous and borderline tumors (36).

The PI3K pathway regulates survival, proliferation, motility, angiogenesis, glucose metabolism, and drug resistance. Activation of the PI3K pathway is observed in 70% of ovarian cancers through multiple mechanisms including amplification or activating mutation of the PI3K p110 α , activating mutation of the PI3K p85, inactivating mutation of the PTEN phosphatase and amplification of AKT2 (Table 34-3). The p110 subunit of PI3K is amplified in 9% to 22% of ovarian cancers, overexpressed in 32%, and mutated in 9% to 12% (37). The pattern of mutation is histology dependent, being common in endometrioid and clear cell tumors but rare in serous cancers. The AKT serine-threonine kinase is generally activated physiologically by the products of PI3K. In 12% to 27% of ovarian cancers the AKT2 kinase gene is amplified and exhibits an increase in AKT kinase activity (38). The p70S6 kinase is downstream of both PI3K and AKT kinase in the signaling cascade. The p70S6 kinase may have a critical role in modulating drug resistance in that rapamycin, an inhibitor of p70S6 kinase, can potentiate sensitivity of some ovarian cancer cell lines to cisplatin-induced apoptosis (39). Inhibition of activated PI3K decreases cell growth, induces apoptosis, and potentiates paclitaxel chemotherapy.

Normal ovarian surface epithelium expresses small amounts of macrophage colony stimulating factor (M-CSF, CSF-1), but little, if any of *fms*, the tyrosine kinase receptor for this ligand. At least 70% of ovarian cancers express and secrete substantially greater amounts of M-CSF and approximately 50% of cancers express the *fms* receptor (40). Interaction of M-CSF with *fms* stimulates invasiveness and up-regulates urokinase-like plasminogen activator (uPA). Expression of uPA correlates with tumorigenicity of ovarian cancer cell lines in xenograft models (41).

Among the nonreceptor kinases, the SRC tyrosine kinase can be physiologically activated in ovarian cancer cell lines, but the *src* gene is rarely amplified or mutated (42). BRK, another cytoplasmic tyrosine kinase, maps to an amplicon on 20q13.3 and is overexpressed in 90% of high-grade serous ovarian cancers (43).

Stat3 is phosphorylated by the Janus kinase (JAK) and Src kinase. Phosphorylated Stat3 forms dimers that can be translocated to the nucleus, binding DNA and inducing transcription of genes required for proliferation (cyclin D, *c-Myc*, *c-Fos*), survival (Bcl-2, Bcl-XL, XIAP, survivin) and angiogenesis (VEGF). Stat3 can be activated on the intracellular domains of peptide growth factor receptors (EGFR) and cytokine receptors (IL-6R) or by direct interaction with either Src or Abl. Activated pStat has been detected in 86% of 322 ovarian cancers (44). Nuclear localization of pStat3 has been observed in 71% of cases, associated with decreased overall survival. Autocrine

and paracrine stimulation with IL-6 activates Stat3, increasing both proliferation and motility. Inhibition of JAK inhibits IL-6–stimulated chemotaxis toward serum and haptotaxis toward fibronectin (45). Knockdown of Stat3 with siRNA inhibits motility and prevents translocation of Stat to focal adhesions. The imprinted tumor-suppressor gene *ARHI* inhibits proliferation and motility, binds to Stat3, and sequesters it in the cytoplasm, preventing translocation to the nucleus and to focal adhesions (22). In addition, *ARHI* can prevent binding of Stat3 to DNA stat response elements in promoter regions.

The endothelin A peptides (ET-1, ET-2, and ET-3) are potent mitogens for several human tumors. ET-1 and its ETA receptor (ETAR) are overexpressed in primary and metastatic ovarian cancers, providing the potential for autocrine stimulation (46). Interaction with ET-1 also transactivates EGFR, stimulates proliferation, blocks apoptosis, activates integrin-like kinase (ILK), up-regulates matrix metalloproteinases (MMPs), and increases vascular endothelial growth factor (VEGF) expression, enhancing angiogenesis. Clinical trials targeting ET-1 are under way.

Lysophosphatidic acid (LPA) is produced constitutively by mesothelial cells and some ovarian cancer cells and accumulates at high levels in the ascites of nearly all ovarian cancer patients. LPA can be detected in the plasma of most patients at all stages of disease. The LPA-2 and LPA-3 receptors are markedly up-regulated in ovarian cancers (47). Interacting with these receptors, LPA stimulates calcium influx, proliferation, motility, chemotaxis, invasion, and resistance to chemotherapeutic agents, signaling through the RAS/MAP and PI3K pathways. LPA is a potent inducer of VEGF, IL8, IL6, and gro, implicating LPA in the accumulation of ascites, neovascularization, and metastasis. Both the enzyme producing LPA, autotaxin, and the enzymes degrading LPA (LPPs) are aberrant in ovarian cancer. Drugs targeting LPA production and action are potent inhibitors of metastasis.

All three TGF- β isoforms are expressed by normal ovarian surface epithelial cells and regularly inhibit their proliferation, maintaining autocrine growth inhibition (48). Expression of TGF- β or loss of responsiveness to the growth inhibitory factor is detectable in a fraction of ovarian cancers. Moreover, TGF- β can stimulate motility and invasiveness of transformed cells. While mutation in Smad4 is observed in a fraction of ovarian cancers, both TGF- β R1 and TGF- β R2 receptors are generally intact, as is Smad signaling. Loss of growth inhibition and increased invasiveness may relate to *EVI-1* overexpression that is observed in 43% of ovarian cancers (49) and is thought to inhibit transcription of TGF- β –responsive genes. SnoN and AML1, which bind to and regulate Smads, are also aberrant in ovarian cancer.

Müllerian inhibition substance (MIS) bears homology to TGF- β , binds to a receptor with similar structure and function, and is produced by the Sertoli cells of the testis and granulosa cells of the ovary (50). During embryonic development of gonadal structures, MIS induces atrophy of the Müllerian duct in male mammals. MIS inhibits growth of human epithelial ovarian cancer cells in culture and in xenografts. Binding of MIS to MISII G-protein–coupled receptors up-regulates p16, produces G₁ cell cycle arrest through an Rb-independent mechanism, and induces apoptosis. Some 56% of human ovarian cancers express MISII

receptors and clonogenic growth can be inhibited with MIS in more than 80% of cancers bearing receptors. Both a “side” population of putative stem cells and the nonside population responded to MIS (51). MIS enhances the anticancer activity of suboptimal doses of chemotherapeutic agents against human and murine ovarian cancer cell lines in culture and as xenografts (52).

Adhesion, Invasion, Metastasis, and Angiogenesis

Several molecular alterations may contribute to the distinctive pattern of metastasis observed in ovarian cancer. Normal ovarian surface epithelial cells bind to laminin, as well as to collagen associated with the basement membrane (53). Adhesion to collagen appears to be maintained in ovarian cancer cells. In cell culture, ovarian cancer explants and cell lines bind preferentially to type I collagen through $\alpha_2\beta_1$ integrin. Alteration in adhesion to laminin may be an important step in ovarian oncogenesis. The β_1 , α_2 , and α_3 integrins can be detected over the entire surface of normal and transformed ovarian epithelial cells. In the normal ovary, α_6 and β_4 integrins are detected only on the basal surface of epithelial cells at sites of binding to basement membrane, whereas solid tumors exhibit only focal expression of these integrins. Ascites tumor cells have markedly decreased expression of α_6 and β_4 , consistent with the possibility that down-regulation of integrin expression could contribute to the release of tumor cells from the basement membrane. The interaction of integrins with the extracellular matrix controls activation of the FAK, ILK, and PI3Ks. These, in turn, play critical roles in determining whether cells undergo apoptosis or anoikis on dissociation from the substratum. In addition to changes in integrin profile, ovarian cancer cells are relatively deficient in E-cadherin when compared with normal ovarian surface epithelium.

Epithelial-mesenchymal transition (EMT) occurs during metastasis in many carcinomas including those that arise in the ovary. Increases in the transcription factors Slug and Snail are associated with loss of intercellular adhesion, as well as specific repression of adherens junction components (E-cadherin and B-catenin), tight junction components (occludin and ZO-1), desmosomal junction components (Dsg2) and neutrophil gelatinase-associated lipocalin (NGAL; 54). N-cadherin and vimentin are increased and Rac1, Rho A, and cdc42 GTPases are activated, as ovarian cancer cells assume a spindle shape and become more motile. EMT can be driven by endothelin A, EGF, LPA, Rab25, bone morphogenic protein-4 (BMP4), hypoxia, and 17- β -estradiol in ovarian cancer cells.

The distinctive pattern of metastasis to the peritoneal surface involves binding of ovarian cancer cells to peritoneal mesothelial cells, mediated by B1 integrin, CD44, and MUC16 (CA125) that are found on the surface of ovarian cancer cells (55). Mesothelial cells express fibronectin, laminin, and type IV collagen that bind to integrins containing the B1 subunit. Incubation of ovarian cancer cells with antibodies against B1 or with each of the matrix proteins blocks adherence of some, but not all ovarian cancer cell lines to mesothelial monolayers.

Mesothelial cells also express hyaluronic acid. CD44, the hyaluronic acid receptor, is synthesized predominantly as a

standard (CD44S) form in normal ovarian surface epithelium, but more than 70% of ovarian cancers exhibit a diverse mixture of CD44 splice variants (56). Anti-CD44 antibodies can partially block adhesion of ovarian cancer cells to peritoneal mesothelial cells and can reduce the frequency of peritoneal metastases (57), suggesting that interaction of CD44 with hyaluronic acid may also be important for tumor cell adhesion and formation of peritoneal implants. Ezrin, part of the submembrane linking complex that connects CD44 to the cytoskeleton, is strongly expressed in 49% of ovarian cancers and is associated with reduced overall survival (58). Knock-down of ezrin with siRNA inhibits invasiveness. Interestingly, ascites tumor cells have decreased CD44 expression.

Adherence of ovarian cancer cells to the peritoneum can also be facilitated by the interaction of MUC16 on the surface of ovarian cancer cells with mesothelin, a glycosylphosphatidylinositol anchored glycoprotein expressed on the surface of mesothelial cells. MUC16, expressed by 80% of ovarian cancers, has a high-molecular-weight (>1 million D) highly glycosylated mucin with a cytoplasmic tail and contains multiple repeating subunits of 154 amino acids in the extracellular domain (59). MUC16 peptides associate with mesothelin and binding of ovarian cancer cells to mesothelial cells can be blocked with antimesothelin antibodies (60). Interaction appears to depend on interaction with N-glycans associated with MUC16 (61). Knock-down of MUC16 decreases the invasiveness of ovarian cancers.

Shed MUC 16 (CA125) has provided a serum biomarker for monitoring the course of ovarian cancer during treatment (62). Increases or decreases in CA125 have correlated with disease course in more than 80% of patients with elevated serum levels of the marker. CA125 has been used to distinguish malignant from benign pelvic masses, identify persistent disease, and detect disease recurrence. Rising CA125 is being evaluated as an initial step to identify patients who would benefit from transvaginal sonography for detection of early-stage ovarian cancer (63). Interestingly, shed mesothelin complements CA125 for detecting women with early-stage ovarian cancer (64).

The ability of tumor cells to migrate and to invade matrigel membranes is increased by VEGF, EGF, heregulin, TGF- β , BMP4, hepatocyte growth factor, M-CSF, TNF- α , heregulin, and LPA. Stress hormones, including epinephrine, norepinephrine, and cortisol have been shown to up-regulate MMP-2 and MMP-9 in ovarian cancer cells, enhancing invasion and angiogenesis (65). Signaling through RAS/MAP, PI3K/AKT/p70S6K and Stat pathways have been implicated in migration and invasion. Effector proteases, including MMP2, MMP7, MMP9, IGFBP2, uPA, and the kallikreins, have all been associated with ovarian cancer cells in culture and in pathologic specimens. MMP-2 and MMP-9 are associated with very early dysplastic lesions where basement membrane is breaking down (66). Ovarian stromal cells are an important source for several of the proteases including MMP-9, which has been associated with infiltrating monocytes.

LPA, generated by phospholipase A2 in mesothelial cells (67), is found at high concentrations in ascites fluid and at detectable levels in the plasma of most ovarian cancer patients. LPA stimulates chemotaxis, adhesion to collagen, and invasion (67), activating

RhoA and ROCK, stimulating FAK autophosphorylation, inducing expression of B1 integrin and increasing the secretion of MMP-2, -7, and -9 as well as uPA (68). LPA also induces VEGF, IL-6, IL-8 and GRO-1 in ovarian cancer cells. IL-6, in turn, mediates autocrine growth stimulation and motility through STAT3; IL-8 promotes angiogenesis; and GRO-1 induces senescence of stromal fibroblasts, which then enhance ovarian cancer growth (69). SPARC expression significantly attenuates LPA-induced proliferation, chemotaxis, and invasion, inhibiting IL-6 secretion and ERK1/2, AKT, and STAT3 signaling (70).

The human kallikreins (hKs) include some 15 different serine-proteases with a high degree of homology that map to a cluster on chromosome 19q13.4. Twelve are transcriptionally up-regulated in ovarian cancer. In aggregate, the kallikreins degrade multiple matrix components including fibronectin, vitronectin, laminin, and collagen I, II, III, and IV. Transfection of hK4, hK5, hK6, and hK7 do not affect proliferation, but increase invasiveness in vitro and formation of peritoneal metastasis in nude mice (71). Kallikrein activity is inhibited physiologically by serpins and antithrombin-3. Several kallikreins are being evaluated as biomarkers for detection or prognostication in ovarian cancer where hK5, hK6, hK7, hK8, hK10, and hK11 are up-regulated and hK14 down-regulated in tissue and in serum (72). Elevated hK5, hK6, hK7, and hK15 have been associated with a poor prognosis and elevated hK8 and hK9 with a good prognosis.

Binding of cancer-associated integrins to extracellular matrix can stimulate chemotaxis and invasion. Adhesion of ovarian cancer cells to collagen and clustering of collagen binding integrins activates integrin-mediated signaling via SRC kinases to induce expression of *EGR1*, resulting in transcriptional activation of the *MT1-MMP* promoter and subsequent MT1-MMP-catalyzed collagen invasion (73). Laminin, fibronectin, and collagen can all enhance chemotactic activity associated with activation of Ras/Map, whereas enhanced invasion is observed only with laminin and collagen. The α_3 , α_6 , and β_1 integrin-mediated signaling through RAS/MAP, Erk, and AKT have been implicated in both processes.

Angiogenesis is an important component of metastatic potential. In primary ovarian cancers, microvessel density has correlated with the propensity to metastasize and with disease-free survival (74). VEGF, IL-6, IL-8, and bFGF are all important for angiogenesis in different ovarian cancers. Acidic FGF-1 has recently been shown to be amplified and overexpressed in ovarian cancers associated with a poor prognosis (75). In human ovarian cancer xenografts, expression of VEGF has correlated with ascites formation and IL-8 content correlated with more aggressive tumor growth. Most ovarian cancers express VEGF, which stimulates proliferation of endothelial cells and serves as a survival factor. Antivascular therapy with the anti-VEGF antibody bevacizumab has produced an objective response rate of 16% in patients with recurrent ovarian cancer and has stabilized disease for 5.5 months in 50% (76). Novel methods for inhibiting VEGF expression are being explored preclinically, including VEGF-Trap and an anti-epHA2 agonist antibody (77). In animal models, treatment with VEGF receptor inhibitors has produced temporary loss of 50% to 60% of tumor vasculature, leaving empty sleeves

with basement membrane and pericytes, along which endothelial sprouts promptly regrow (78). Platelet-derived growth factor (PDGF) has been detected in areas of increased blood flow in ovarian cancer. Pericytes that cover vessels express receptors for PDGF and secrete VEGF, creating a paracrine loop with vascular endothelial cells. Consequently, pericytes might also need to be targeted for effective therapeutic outcomes. Strategies are being evaluated to inhibit both VEGF and PDGF to provide more effective antivasculature therapy.

Immunologic and Inflammatory Factors

Cytokines and Chemokines

Ovarian cancers can express up to 1,000 times more TNF- α than normal ovarian epithelial cells. Some 80% of ovarian cancers express TNF- α that is regulated translationally and transcriptionally through NF- κ B. Ovarian cancer cells can also express both TNFRI and TNFRII receptors that permit both autocrine and paracrine stimulation. Exogenous TNF- α or IL-1 α enhances the expression of endogenous TNF- α and increases levels of IL-1 α , IL-6, CCL2, CXCL8, and M-CSF (79). TNF- α can exert contrasting effects on different ovarian cancers, by inhibiting, failing to affect or stimulating tumor cell proliferation. In 10% to 25% of tumor cells taken directly from patients, TNF- α can stimulate clonogenic growth. Knock-down of endogenous TNF- α has inhibited the growth and dissemination of ovarian cancer xenografts (80). Clinical trials have been undertaken with the infliximab in ovarian cancer patients.

Immunosuppression

Immunodeficiency has been documented in patients with ovarian cancer. Advanced disease has been associated with defects in delayed cutaneous hypersensitivity and the humoral immune response. Prior to treatment, T-cell numbers and subsets in peripheral blood have been comparable to controls, but functional defects of B cells have been detected (81). T cells isolated from ascites fluid or tumor tissue exhibit decreased expression of the TCR-zeta chain and downregulation of TCR-zeta chain can be produced *ex vivo* by coculture of T cells with macrophages or soluble tumor-derived factors (82). The presence of CD4+CD25+FOXP3+ regulatory T cells suppresses specific T-cell-mediated immunity in tumor masses but not in stroma and has been associated with decreased survival (83). Tumor cells and micro-environmental macrophages produce the chemokine CCL22, which mediates trafficking of Treg cells. Ovarian cancers can also produce TGF- β and several immunosuppressive factors including IL-10, fibronectin, and mucins.

Humoral Immune Response

Despite an immunosuppressive environment, antibodies against tumor-associated antigens can be found in the blood of ovarian cancer patients. Correlation of MUC1 antibodies with favorable risk factors has raised the interesting hypothesis that

immunity to mucins might suppress development of ovarian cancers, although there may be many different reasons for anti-MUC1 antibodies (85). Antibodies against mesothelin, p53, and HER2 have been found in a minority of ovarian cancer patients.

Cellular Immune Response

T cells from ovarian cancer patients can kill autologous tumor cells following *in vitro* activation. Cytotoxic T cells can be generated by incubating peripheral blood lymphocytes with dendritic cells that have been pulsed with extracts of autologous, but not allogeneic ovarian cancers (84). Cytotoxic T cells bear Fas ligand and induce apoptosis in cells that express Fas, which includes most ovarian cancers. Restricted expression of T-cell receptor V- β subtypes has been observed in tumor-associated lymphocytes consistent with antigen-driven expansion of specific clones. Aberrantly glycosylated mucins, including MUC1, are expressed by most ovarian cancers, and T cells reactive with mucin molecules have been obtained from ovarian cancer patients. p53 is mutated in approximately 70% of ovarian cancers and T cells reactive with TP53 can be detected in some 50%, but are also found in a similar fraction of controls with benign disease (85). T cells reactive with HER-2/*neu* epitopes have been isolated from the ascites fluid of ovarian cancer patients.

As T-cell antigen epitopes are recognized in the context of specific major histocompatibility complex (MHC) determinants. Normal ovarian surface epithelial cells express class I, but not class II MHC components. In ovarian cancers, class I determinants are expressed in approximately 80%, and class II determinants expressed in 40%. The level of class I MHC expression in epithelial ovarian cancer cells has correlated with the degree of T-cell infiltration *in vivo* and the ability to expand T cells *in vitro* in the presence of low levels of IL-2. Low class I MHC expression is a poor prognostic factor in aneuploid ovarian cancers. Antibodies reactive with autologous tumor cells have also been identified.

Ascites contains widely varying fractions of lymphocytes, macrophages, mesothelial cells, and cancer cells. In one study, an average of 51% CD8 T cells, 10% CD4 T cells, and 27% CD14 macrophages were encountered (86). A variety of chemokines—CCL-2, 3, 4, 5, 8, and 22—and their receptors—CCR1, 2a, 2b, 3, 4, 5, and 8—were detected and a direct correlation was found between CCL5 and the CD3 T-cell infiltration. Chemokine CXCL12 and its unique receptor CXCR4 have been implicated in metastasis of several different cancers. CXCL12 was found in 91% of ovarian cancers and CXCR4 in 59% (87). Expression of CXCR4 was associated with decreased disease-free and overall survival. CXCL12 and VEGF are both induced in ovarian cancer cells by hypoxia and synergize to induce tumor vessels (88). CXCL12 also attracts plasmacytoid dendritic cells into ascites that further enhance angiogenesis by secreting IL-8 and TNF- α (89).

Among the cells that infiltrate solid ovarian cancers, T cells are most prevalent, B cells are rare, and macrophages vary

in number. In addition to the specific cytotoxic effects of T cells, the interferons produced by activated T cells can inhibit tumor growth, inhibit IL-8 secretion, block angiogenesis, up-regulate MHC, and augment mucin expression. Intratumoral T cells have been found in 55% of ovarian cancers and are associated with a 5-year survival rate of 38%, compared with 4.5% for patients whose tumors lack T-cell infiltrates (90). Both CD4 and CD8 cells can be found at tumor sites. The presence of CD8 T cells and a high CD8/CD4 ratio has correlated with the most favorable prognosis, related to the adverse effect of CD4+CD25+FOXP3+ regulatory T cells within the CD4⁺ population (91). Ovarian cancer cells can secrete M-CSF and MCP1 that exert potent chemotactic activity for macrophages. Cytokines and factors released from activated macrophages can stimulate (IL-1, IL-6, TNF) or inhibit (nitric oxide, TNF) tumor growth. Tumor-associated macrophages have impaired phagocytic activity and effector function for ADCC. Cytotoxic NK cells have been detected in ascites fluid and in solid ovarian cancers. Despite these many potential immune effector mechanisms, most ovarian cancers grow progressively.

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Conclusion

Much has been learned over the last two decades regarding the cellular and molecular biology of ovarian cancers. Development of different histotypes, the formation of ascites, and mechanisms of peritoneal implantation are better understood. Genomic instability leads to remarkable heterogeneity among cancers from different women. Genetic and epigenetic alterations in oncogenes, putative tumor-suppressor genes, RNA splicing, and miRNA produce aberrant signaling through several pathways, including PI3K and Ras/Map. The microenvironment of most ovarian cancers is generally hypoxic and rich in angiogenic factors (VEGF, IL-6, IL-8, bFGF), growth factors (LPA, IGF), and cytokines (TNF and M-CSF). Macrophages and T cells can influence ovarian cancer growth. Drug resistance arises from multiple mechanisms. Late detection and the ultimate growth of dormant, drug-resistant cancer cells remain the clinical challenges. Multiple targets for early detection and for more specific and selective therapy have been identified and will undoubtedly contribute to more effective management of ovarian cancer.

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Molecular Pathogenesis of Pancreatic Adenocarcinoma

The most common exocrine pancreatic neoplasm is pancreatic ductal adenocarcinoma, which accounts for more than 95% of all pancreatic malignancies. Other pancreatic malignant neoplasms include acinar cell carcinoma, serous cystadenocarcinoma, mucinous cystadenocarcinoma, intraductal papillary-mucinous neoplasm, osteoclast-like giant cell tumor, solid pseudopapillary carcinoma, and pancreatoblastoma.

It has been hypothesized that development of pancreatic adenocarcinoma follows progressive stages of neoplastic growth through precursor lesions to adenocarcinoma, similar to the models proposed for colorectal cancer and prostate cancer. The precursor lesions are best defined for invasive ductal adenocarcinoma and have been termed “pancreatic intraepithelial neoplasia” (PanIN). PanINs are characterized by architectural changes manifest by change of the normal cuboidal epithelium to a columnar epithelium, nuclear hyperchromasia and atypia, loss of epithelial polarity, pseudostratification and papillary folding, and eventually to carcinoma in situ and invasive carcinoma (Figure 35-1; 1).

Molecular Alterations in Pancreatic Cancer

Pancreatic carcinogenesis is driven by multiple genetic and epigenetic events, including inactivation of tumor-suppressor genes and activation of proto-oncogenes. Table 35-1 shows a list of the most frequently identified genetic alterations. *K-ras* mutation is believed to be an early genetic event, followed by loss of functional p16, p53, SMAD4, and many other changes. This chapter focuses on some of the key molecular changes that characterize exocrine pancreatic cancer (2).

Events Targeting Signal Transduction Pathways

K-ras Mutation

K-ras mutations can be detected in approximately 30% of early PanINs and increase in frequency with disease progression. *K-ras* mutations can be identified in nearly 95% of invasive ductal

pancreatic adenocarcinomas (3). The early onset of *K-ras* mutations suggests a role in tumor initiation. Transgenic mice models have provided further evidence that support this notion. A first-generation transgenic mouse model was generated in which *K-ras* was driven by an elastase promoter and the resultant transgene was expressed in pancreatic acinar cells. Transgenic mice bearing this transgene exhibit acinar hyperplasia, acinar-ductal metaplasia, and noninvasive intrapapillary mucinous neoplasia (IPMN). Frequently, these lesions were accompanied by focal dysplasia, fibrosis and/or lymphocytic infiltration, and occasional carcinoma in situ (4). All lesions appear arrested at a preinvasive stage despite confirmed expression of the *K-ras* gene, suggesting that in this model *K-ras* mutation is not sufficient to induce progression to invasive pancreatic carcinoma and that other genetic events are required. A similar mouse model was developed in which the cytokeratin 19 promoter, specifically active in pancreatic ductal cells but not other cell types, was engineered with mutant *K-ras*. Mice bearing *K-ras* mutations driven by the cytokeratin 19 promoter exhibited specific phenotypic changes, including periductal lymphocytic infiltration in the pancreata of transgenic mice, mucous neck cell hyperplasia, occasional evidence of pancreatic ductal hyperplasia similar to human PanIN, and induction of N-cadherin (5). N-cadherin is known to serve as a checkpoint to the proliferation-mediated and growth-promoting effects of activated ras. Therefore the induction of N-cadherin expression and periductal lymphocytic infiltration were suggested to counterbalance the crucial initiating events in pancreatic carcinogenesis precipitated by *K-ras*. However, neither of these models demonstrated clear progression to invasive adenocarcinoma typical of human pancreatic cancer.

To overcome these effects, a second mutation, such as inactivation of tumor-suppressor genes, p16, SMAD4, and/or p53 may be necessary for the development of invasive/metastatic pancreatic adenocarcinoma. Consistent with this hypothesis, more recent transgenic models have used genetically engineered mice with promoters that are developmentally expressed in progenitors of all pancreatic cell types. These genetically engineered mice expressing the mutant *K-ras*^{G12D} allele mutation develops focal premalignant lesions consistent with human PanIN (6), but a mouse with activation of a mutant *K-ras* allele (*Kras*^{G12D}) and deletion of a conditional *Ink4a/Arf* tumor suppressor allele resulted in an

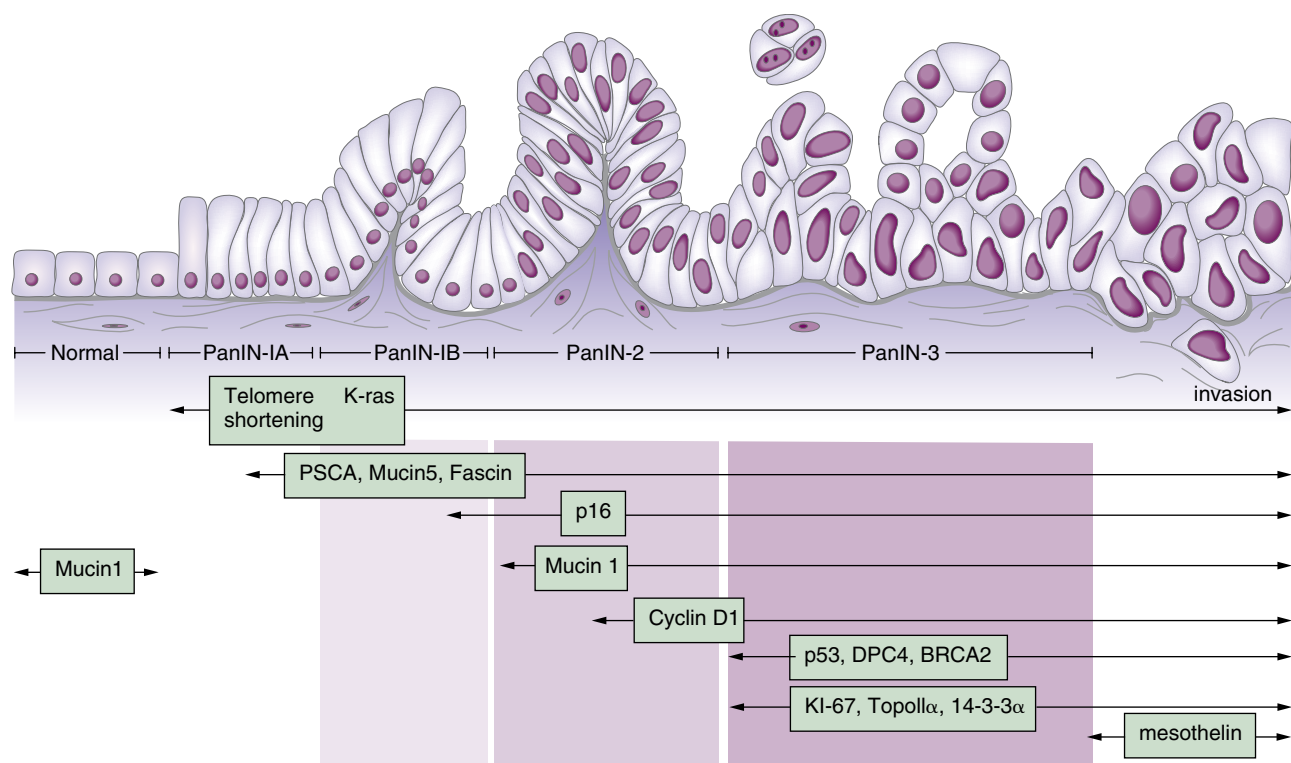


FIGURE 35-1 Pancreatic epithelial neoplasia and the multistep model of exocrine pancreatic cancer. (From Maitra A, et al. Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. *Mod Pathol* 2003;16:902, with permission.)

earlier appearance of PanIN lesions, and these neoplasms progressed rapidly to highly invasive and metastatic cancers (7). The evolution of these tumors bears striking resemblance to the human disease, possessing a proliferative stromal component and ductal lesions with a propensity to advance to a poorly differentiated state. These findings in the mouse provide experimental support for the widely accepted model of human pancreatic adenocarcinoma in which activated *K-ras* serves to initiate PanIN lesions, and *INK4A/ARF* or other tumor suppressors function to constrain the malignant conversion of these PanIN lesions into lethal ductal adenocarcinoma.

Table 35-1 Frequent Molecular Alterations in Pancreatic Adenocarcinoma

Molecular Alteration	Frequency of Event in Exocrine Pancreatic Cancer, %
Oncogene activation	
<i>K-ras</i>	90
RTK overexpression	
EGFR	95
HER2	10
Tumor suppressors	
P16 ^{INK4A}	95
P53	55
SMAD4	50
PTEN	60
Transcription factor activation	
NF-κB	67

RTK, receptor tyrosine kinase.

Although *K-ras* mutation appears critical to the initiation of pancreatic carcinogenesis, its importance in established pancreatic adenocarcinoma is not clear. In addition, although *K-ras* mutation is widely detected in pancreatic adenocarcinoma, its expression can also be detected in non-malignant conditions such as chronic pancreatitis. Disappointingly, novel therapies that target mutant *K-ras* have not been effective.

Epidermal Growth Factor Receptor

The role for epidermal growth factor receptor (EGFR) and its downstream signaling molecules in tumorigenesis is evidenced by their ability to transform normal cells to a neoplastic phenotype when expressed in mutated, unregulated forms or when expressed to an abnormally high level. Overexpression of EGFR and its downstream signaling molecules occurs frequently in a variety of human cancers, including pancreatic cancer. A prospective study indicated that EGFR was detectable in more than 95% of patients with advanced pancreatic cancer (8). In most cases, EGFR is concomitantly expressed with its ligands, EGF or TGF-α. It has been hypothesized that the increased expression of ligand and receptor forms an autocrine loop that constantly stimulates cell proliferation. A study found that pancreatic cancer cells lines display heterogeneous sensitivity to the EGFR inhibitor gefitinib. Three of nine cell lines studied displayed significant sensitivity to pharmacologically and clinically relevant concentrations of gefitinib (1 μmol/L) as measured by two independent assays for G₁-S cell cycle arrest (9). There was a linear correlation between gefitinib sensitivity and TGF-α expression in these cell lines, strongly suggesting that autocrine TGF-α

production drove EGFR pathway dependency. In addition, when the gefitinib sensitivities of parental L3.6pl cells (a pancreatic cancer cell line with high metastatic potential to liver) and subclones of L3.6pl that constitutively produced lower levels of TGF- α were compared, the parental cells were much more sensitive to gefitinib-mediated growth arrest than the subclones. Finally, using a siRNA construct directed against TGF- α , it was confirmed that a causal relationship exists between cellular TGF- α production and EGFR pathway activation and proliferation in the L3.6pl cells. These results suggest that TGF- α expression identifies those human pancreatic cancer cell lines that exhibit constitutive EGFR pathway activation and are likely to be sensitive to EGFR antagonists in vitro. These data would predict that in the clinic EGFR inhibitors such as gefitinib or erlotinib may be effective against a subset of pancreatic adenocarcinoma that displays TGF- α -dependent EGFR activation.

Activation of Nuclear Transcription Factors

Nuclear Factor κ B

Nuclear factor κ B (NF- κ B) is a family of pleiotropic transcription factors that regulate the expression of a spectrum of genes important in growth, oncogenesis, differentiation, and apoptosis. NF- κ B proteins are normally sequestered in the cytoplasm in an inactive form through their association with the inhibitor I κ B α , which masks the nuclear localization signal (NLS) of NF- κ B, thereby preventing its nuclear translocation. NF- κ B is activated through activation of the I κ B kinase complex (IKK), which phosphorylates I κ B α and as a result of proteasomal degradation releases NF- κ B from the complex exposing the NLS. Constitutive NF- κ B activation has been detected in approximately 70% pancreatic adenocarcinoma and nine of 11 human pancreatic tumor cell lines, but not in normal pancreatic tissue (10).

Many upstream events can potentially activate NF- κ B. Pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α) and interleukin 1 induce rapid degradation of I κ B α resulting in nuclear translocation of NF- κ B and sustained activation of NF- κ B mediated through I κ B β . The epidermal growth factor receptor (EGFR)-mediated signaling cascade and Ras signaling pathways may induce constitutive NF- κ B activity as well.

Once activated, NF- κ B mediates transcription of numerous genes encoding growth factors, cytokines, and apoptotic and cell cycle regulators. The expression of the apoptosis inhibitors that are regulated by NF- κ B include c-IAP1, c-IAP2, Traf1, Traf2, A20, IEX-1, and the Bcl-2-homologues Bfl-1/A1 and Bcl-x. NF- κ B also activates the expression of genes that are important in invasion and metastasis, including matrix metalloproteinases, urokinase plasminogen activator, and ICAM-1. Like NF- κ B, numerous other nuclear transcription factors such as AP1 (11), Sp proteins (12,13), and Stat3 (14) have been shown to be activated in exocrine pancreatic cancer. In some cases these proteins are being actively targeted for therapy (15).

Loss of Tumor Suppressors

INK4A and ARF Tumor Suppressors

Studies have demonstrated that the 9q21 locus encodes two important and overlapping tumor suppressor proteins: p16^{INK4A}

and p19^{ARF}. Loss of p16^{INK4A} appears to be critical for the development of exocrine pancreatic cancer occurring in up to 95% of cases (16). Genetically, loss of the INK4A locus can occur through mutation, deletion, or promoter hypermethylation. Functionally, loss of p16^{INK4A} allows CDK4/6 to phosphorylate RB thereby facilitating entry into the S-phase of the cell cycle. The importance of p16^{INK4A} in pancreatic carcinogenesis has been highlighted by studies of Bardeesy et al. (17) where p16^{INK4A} mutations cooperate with mutant K-ras and p53 mutations in the development and progression of exocrine pancreatic carcinomas.

p53

The p53 tumor suppressor is mutated in at least 50% of patients with exocrine pancreatic cancer and appears to be most important during later stages of tumor progression (18). Loss of p53 may also contribute to drug resistance and the chromosomal instability that are characteristic of pancreatic cancer.

SMAD4

SMAD4 is a tumor suppressor gene (19). Smad proteins belong to a family of proteins that are part of TGF- β signaling pathway which negatively regulate the growth of epithelial cells. Upon binding of TGF- β , TGF- β receptor II activates TGF- β receptor I by phosphorylation. TGF- β receptor I in turn activates Smad2 and Smad3. The activated Smad2 and Smad3 form a hetero-oligomer with Smad4. This Smad complex translocates to the nucleus where it interacts with DNA directly or indirectly through other DNA-binding proteins, regulating transcription of target genes. SMAD4, also known as DPC4 (homozygously deleted in pancreatic carcinoma locus 4), is frequently deleted or mutated in pancreatic carcinoma. Nearly 90% of pancreatic carcinoma cases show loss of heterozygosity for SMAD4 and 30% to 37% homozygous deletion of the SMAD4 region. In addition there are intragenic inactivating mutations, including nonsense, missense, and frame-shift mutations. In total, approximately 55% of pancreatic carcinomas have deletion or an inactivating mutation of SMAD4. Loss of SMAD4 occurs with a frequency of 10% or less in other malignancies, which suggests a specific role for SMAD4 in pancreatic carcinogenesis (20). Inactivation of SMAD4 gene correlates with loss of expression of its protein and can be monitored during progression of PanINs. In one study, of 188 PanIN lesions examined, Smad4 was not expressed in 31% of the high-grade lesions, whereas all low-grade PanIN lesions had detectable Smad4 protein (21). This observation is consistent with the notion that K-ras is the initiation factor in pancreatic carcinogenesis followed by alterations of a variety of genes, including SMAD4, p16, p53, and others.

SMAD4 may also be a prognostic factor. Using immunohistochemistry, the SMAD4 protein status of 249 pancreatic adenocarcinomas from patients who underwent pancreaticoduodenectomy was examined. The SMAD4 gene status of 56 (22%) of 249 pancreatic carcinomas was also determined. It was found that patients with pancreatic adenocarcinomas with Smad4 protein expression had significantly longer survival (unadjusted

median survival was 19.2 months as compared with 14.7 months in patients with pancreatic cancers lacking Smad4 protein expression; $p = 0.03$). This Smad4 survival benefit persisted after adjustment for other known prognostic factors including tumor size, margins, lymph node status, pathologic stage, blood loss, and use of adjuvant chemoradiotherapy (22).

PTEN

The tumor suppressor gene *PTEN* is known to play a major role in embryonic development, cell migration, and apoptosis (23). *PTEN* acts as a lipid phosphatase that regulates major signal transduction pathways and effectively inhibits phosphatidylinositol-3-kinase (PI3K)-mediated signaling. *PTEN* mutation, which occurs frequently in many solid tumors, is associated with constitutive activation of the PI3K/Akt pathway, resulting in tumors that are generally resistant to apoptosis. In pancreatic cancer, *PTEN* is not mutated but functionally abrogated through loss of expression. It was found that over 60% of pancreatic cancer cell lines and tumor tissues had decreased or loss of expression of *PTEN* (24). The role of *PTEN* in pancreatic carcinogenesis was also studied using a pancreas-specific *PTEN* knock-out mouse model (25). Knock-out mice display pancreatic ductal metaplasia as the predominant phenotype and occasional PanINs. These lesions are characterized by progressive replacement of the acinar pancreas with highly proliferative ductal structures that contain abundant mucins and express Pdx1 and Hes1, two markers of pancreatic progenitor cells. A fraction of these mice develop ductal malignancy. Further studies showed that ductal metaplasia resulted from the expansion of centroacinar cells rather than transdifferentiation of acinar cells into ductal cells. These results suggest that *PTEN* actively maintains the balance between different cell types in the adult pancreas and that misregulation of the *PTEN* pathway in centroacinar cells may contribute to the initiation of pancreatic carcinoma in vivo.

Reactivation of Developmental Biology Pathways

Hedgehog, Notch, and Wnt Pathways

The relationship between developmental pathways for pancreatic organogenesis and pancreatic cancer has recently gained in appreciation (26). The hedgehog (Hh) family of genes—sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh)—encode signaling molecules that regulate multiple functions during organ development and in adult tissues. Altered hedgehog signaling has been implicated in disturbed organ development as well as in different degenerative and neoplastic human diseases. Hedgehog signaling plays an important role in determining the fate of mesoderm in the primitive gut tube, as well as in early pancreatic development and islet cell function. Multiple groups have reported that the hedgehog, Notch, and Wnt developmental cascades might be reactivated during the development of pancreatic cancer through the up-regulated expression and/or activation of these complex signaling pathways. The hedgehog signaling molecules, Shh, Ihh, Ptc, Smo,

and Gli1, are frequently overexpressed in pancreatic cancer tissues and cell lines as well as in PanIN lesions (27). Specific inhibition of Hh activity in pancreatic cancer cells using cyclopamine can reduce pancreatic cancer cell growth both in vitro and in vivo (Figure 35-2). The reduction of the proliferative activity of pancreatic cancer cells is mediated through G_0/G_1 cell cycle arrest in vitro or induction of apoptosis in vitro and in vivo. Similarly, the reactivation of the Notch pathway in epithelial cells seems to contribute to initiation of pancreatic tumorigenesis at the earliest phases. Interactions between Notch and *K-ras* signaling could confer a more malignant phenotype to pancreatic epithelial cells through the activation of Notch signaling (28). β -catenin—aberrant nuclear expression is more frequent in high-grade PanIN lesions and in ductal adenocarcinomas than in normal ducts and low-grade PanIN lesions. In addition, defects in the Wnt/ β -catenin signaling through β -catenin stabilization seem also to occur in pancreatic nonductal neoplasms such in solid pseudopapillary tumors and acinar cell carcinomas.

Downstream Events

Desmoplastic Reaction (Tumor Stroma)

One of the morphologic hallmarks of pancreatic adenocarcinoma is its desmoplastic reaction, or tumor stroma. Desmoplastic tissue consists of fibroblasts, as the main cellular component, infiltrating inflammatory and immune cells, endothelial cells, and extracellular matrix (ECM) proteins, such as fibronectin and collagen (29). Pancreatic adenocarcinoma exhibits a threefold increase in interstitial fibrillar collagen (types I and III) compared with the normal pancreas (30,31). The desmoplastic reaction is also associated with proliferation of fibroblastic cells, which in some cases outnumber tumor cells. Evidence suggests that these are mesenchymal cells, known as stellate cells, which have differentiated into an activated myofibroblastic phenotype. These activated myofibroblasts have been identified as the principal source of type I collagen in the desmoplastic stroma (32). Pancreatic stellate cells are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, pancreatic stellate cells are transformed from quiescent phenotypes into highly proliferative myofibroblast-like cells that produce ECM proteins. Activated pancreatic stellate cells are observed in abundance in pancreatic tumor tissue, suggesting that they are responsible for the deposition of matrix components and the desmoplastic reaction that surrounds the pancreatic tumor, although pancreatic tumor cells are capable of producing ECM proteins. Cell culture experiments have demonstrated that pancreatic tumor cells stimulate the growth of pancreatic stellate cells and ECM formation. The growth-stimulating effects are probably mediated by platelet-derived growth factor, fibroblast growth factor 2, and TGF- β 1 secreted by pancreatic cancer cells. On the other hand, pancreatic stellate cells can stimulate the growth of pancreatic cancer cells as demonstrated by an in vivo study, in which co-injection of pancreatic stellate cells and tumor cells subcutaneously produced larger and faster growing tumors than injection of pancreatic tumor cells alone. Pathologic examination of tumor tissues

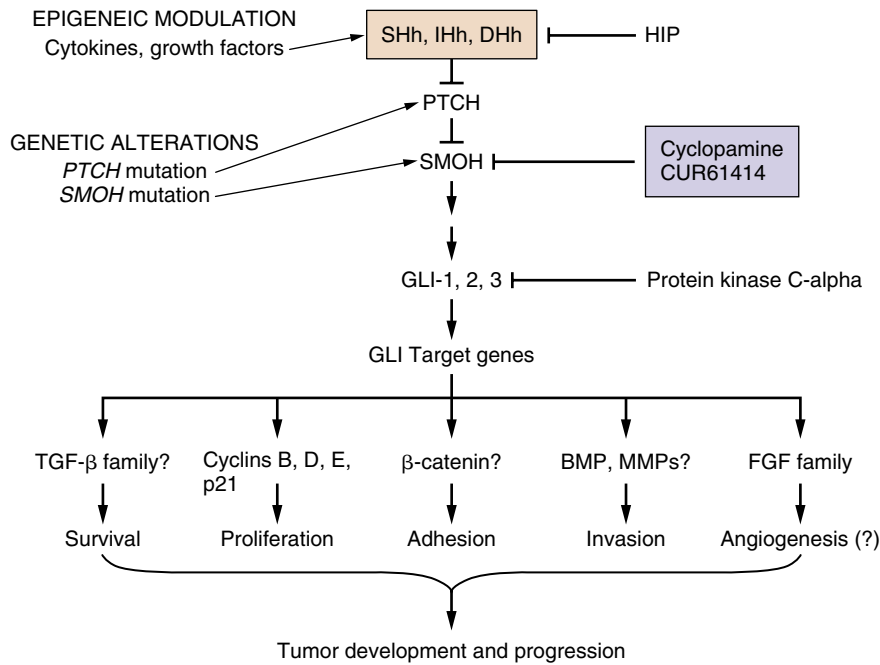


FIGURE 35-2 HEDGEHOG SIGNALING, CARCINOGENESIS AND POTENTIAL THERAPEUTIC TARGETS. Up-regulation of Hh ligands may be mediated by epigenetic events. Mutations in PTCH and SHOH result in activation of hedgehog signaling and are causative in basal cell carcinoma and medulloblastoma. Gli proteins are thought to mediate activation of Hh transcriptional targets potentially important in tumorigenesis, progression, and metastasis. DHh, desert hedgehog; Gli, cubitus interruptus-like transcription factor involved in glioma formation; Hh, hedgehog; HIP, hedgehog interacting protein; IHh, Indian hedgehog; PTCH, patched; SHh, sonic hedgehog; SMOH, smoothened. (From Xie K and Abbruzzese JL. *Developmental biology informs cancer: The emerging role of the hedgehog signaling pathway in upper gastrointestinal cancers.* Cancer Cell 2003;245–247, with permission.)

showed an intense desmoplastic reaction in tumors developed after injection of pancreatic stellate cells and tumor cells (33).

TGF- β is one of the major growth factors stimulating the growth of pancreatic stellate cells. Evidence indicates that the predominant source of TGF- β may be from infiltrating granulocytes, although pancreatic tumor cells are capable of producing TGF- β (34). Immunohistochemical staining of pancreatic tumor tissue showed that isolated cells, mainly located at the invasive edge surrounding cancerous nests, prominently stained for TGF- β . Those cells contain a segmented nucleus and are negative for anti-macrophage (CD68) and positive for antigranulocyte antibodies, indicating they are granulocytes (35).

Cytokine Production

Pancreatic cancer is known to secrete growth factors that stimulate cancer growth through paracrine or autocrine mechanisms. In addition pancreatic cancer secretes many cytokines that impact cancer development through interaction with its microenvironment but also affect overall host physiology (36). Patients with pancreatic carcinoma often have elevated circulating levels of interleukin-6 (IL-6), IL-10, IL-8, and IL-1RA compared with the levels in healthy individuals (37). Furthermore, elevation in one cytokine often correlated with elevation in others. For instance, high IL-10 levels were correlated with high IL-8 and high IL-6 levels. IL-6 is a pleiotropic cytokine that has been implicated in the pathogenesis of several lymphoproliferative disorders, including multiple myeloma, lymphoma, and Castleman disease. It was found that high IL-6 levels in patients with pancreatic carcinoma were correlated with worse survival and weight loss (38). Further evidence suggested that IL-6 is involved in the development of cachexia, which is a clinical hallmark of pancreatic carcinoma.

The pathobiology of cachexia is poorly understood; however, IL-6 and other cytokines may contribute to its development (39). In one study, gene chip analysis of resected pancreatic cancer tissue including 5,600 human genes revealed a significant difference between patients with and without cachexia in only four factors: IL-6, neuropeptide Y Y3 receptor, neurotensin, and islet amyloid polypeptide. IL-6 was significantly overexpressed in pancreatic specimens and elevated in the serum of cachectic patients. A coculture system revealed that pancreatic cancer cells can stimulate IL-6 production exclusively from peripheral blood mononuclear cells derived from cachectic patients, and this effect could be reduced by IL-6-neutralizing antibodies. These data indicate that IL-6 may represent a prominent cachexia-associated factor in pancreatic cancer.

IL-8 expression is also frequently elevated in both serum and pancreatic tumor tissue. IL-8 was originally identified as a neutrophil chemotactic factor. As a member of the CXC chemokine family, IL-8 plays an important role in inflammation and inflammation-induced angiogenesis (40). It is now known that IL-8 is produced by a variety of normal and tumor cells. It was found that about 80% of pancreatic cancer lines constitutively express high level of IL-8 in vitro. The role of IL-8 in tumor growth and metastasis has been studied using tumor cell lines, xenograft models, and human tumor tissue (41). Using orthotopic xenograft models that express different levels of IL-8, it was clearly demonstrated that the level of IL-8 expression correlated with local tumor invasion and distant metastasis. Abrogation of IL-8 expression by antisense oligonucleotides inhibited IL-8 expression and consequently tumor growth and metastasis. In addition, decreased microvascular density of tumor lesions was correlated with decreased levels of IL-8.

Conclusion

Exocrine pancreatic cancer remains a challenging disease. Early diagnosis is infrequent, and therapy only has a limited impact on the survival of patients with advanced pancreatic cancer. Despite these ongoing challenges, our understanding of pancreatic

carcinogenesis and the molecular biology of pancreatic cancer has expanded rapidly over the past 5 years. It is anticipated that these advances coupled with the development of biomarkers for early diagnosis will provide the means for early detection of pancreatic cancer and rapidly accelerate the development of effective therapies.

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Molecular Biology of Melanoma

The incidence of melanoma has risen faster than any other cancer in Western countries. In 2006, $\approx 62,000$ new cases are projected in the United States. It is the most life-threatening form of skin cancer, responsible for $\approx 75\%$ of skin cancer deaths. Most melanomas arise in the skin, with $\approx 3\%$ to 5% from the eye and rarely from internal mucosal membranes. Melanocytic lesions, including benign nevi (moles), dysplastic nevi, and primary melanomas, are visible in the skin and can be clinically evaluated and characterized over time. The visibility of melanocytic lesions has made this an excellent model for studying steps in lesion progression, from normal to premalignancy to malignancy. Melanoma can arguably be characterized as the most malignant human cancer, giving rise to metastases in virtually every tissue, and death from melanoma is almost always due to distant metastases.

Melanocyte Biology

Melanocytes and Pigmentation

Melanocytes are specialized cells that are distributed in the skin, other epithelial surfaces and the eye (1). In the skin, melanocytes are typically distributed at infrequent but regular intervals along the basal layer of the epidermis (Figure 36-1) and in hair follicles (2). A primary function of melanocytes is the distribution of packages of the pigment melanin to neighboring keratinocytes. Distribution of pigment is accomplished through the transfer of melanosomes, a unique organelle where the chemical steps in melanin biosynthesis occur (3). These structures are then transferred from the ends of the dendritic processes of melanocytes to adjacent keratinocytes. Melanocytes in the skin are generally long-lived, nonproliferative cells that constitutively express the anti-apoptotic molecule BCL2, but are able to proliferate in response to inflammation during tissue damage or following excessive solar radiation.

Melanocyte Development and Melanocyte Stem Cells

Melanocyte stem cells come from the neural crest (4,5). Multipotent neural crest cells migrate to the skin where they differentiate into melanocytes, but also migrate widely in embryonic tissue to form diverse structures as the peripheral nervous system, cartilage,

muscle, and bone. Migration of neural crest cells involves interactions of cell surface integrins with extracellular matrix, a process also involved in melanoma invasion and metastasis. Furthermore, the fate of neural crest cells is determined by signals through the Wnt and fibroblast growth factor pathways, which down-regulate expression of E-cadherin (6). These pathways and gene products are also implicated in melanoma progression, leading to speculation that the program regulating migration of neural crest cells is reactivated in melanoma, accounting for the efficiency of melanoma to metastasize.

In hair follicles, differentiated melanocytes reside at the base of the hair, whereas second location further up along the hair, called the bulge region, provides the niche for melanocyte stem cells (4,5). One progeny of stem cells maintains the ability for self-renewal and the other gives rise to a more differentiated melanocyte (7). Eventual depletion of stem cells through differentiation or senescence accounts for hair graying during aging (4). Decisions for self-renewal versus differentiation of melanocyte stem cells are determined by a balance between the transcription factors SOX10 (which maintains “stemness”), PAX3, and MITF (8). In particular, MITF is a master regulator of melanocyte differentiation, including melanosome formation and pigmentation (5,9). These same transcription factors are expressed by melanoma. Whether melanoma has a cancer stem cell counterpart with similar biology to the melanocyte stem cell is not clear.

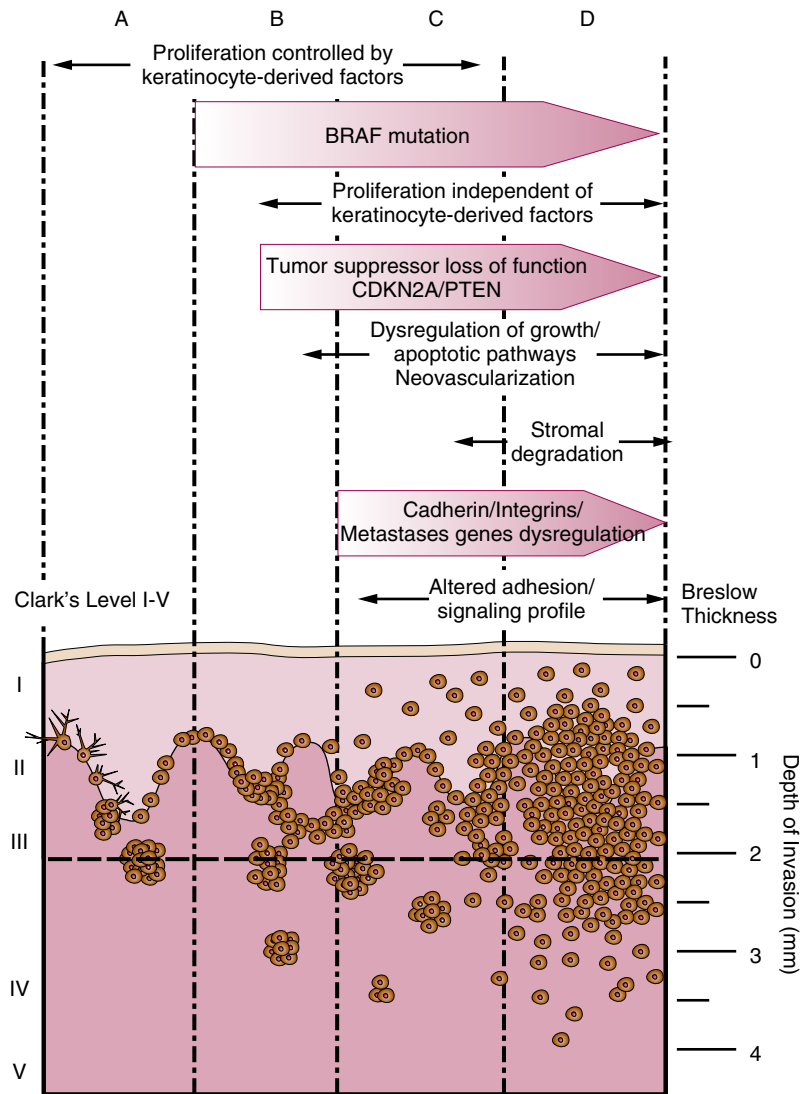
The Epidermal: Melanocyte Unit

The epidermal: melanocyte unit comprises one melanocyte for every ≈ 30 to 50 keratinocytes, the predominant cell type of the epidermis (Figure 36-1). The unit protects the host from potentially damaging sun exposure. Melanin absorbs transferred to keratinocytes protects from potentially damaging ultraviolet (UV) radiation.

Production of Melanin

Melanin is a polymer of variable forms that absorbs light across a broad spectrum of wavelengths, absorbing both UVA (tanning UV wavelengths) and the shorter wavelength, higher energy UVB (sunburn wavelengths; 3). The most common form of

FIGURE 36-1 PROGRESSION OF MELANOCYTIC LESIONS; A MODEL CORRELATING HISTOLOGIC APPEARANCE OF MELANOCYTIC LESIONS WITH BIOLOGIC ALTERATIONS AND MOLECULAR EVENTS: A vertical section of skin is illustrated, with epidermis (pink, top, corresponding to Clark's level I) and dermis (magenta, Clark's levels II–IV) and subcutaneous tissue (magenta, Clark's level V). The Breslow thickness (right) and Clark's levels (left) are microstaging systems used by pathologists to categorize the extent of invasion of a given tumor. Biologic alterations correlating with histologic appearance of melanocytic lesions are indicated with arrows. Molecular events are described in the orange open arrows. See text for further details. **A:** Normal melanocytes at the dermal–epidermal junction are arranged individually (left) or in small nests of benign nevi (right). Some melanocytes are shown with dendritic processes to illustrate the normal function of these cells—the transfer of melanin pigment to surrounding keratinocytes. **B:** Dysplastic changes are shown, including proliferation of melanocytes along the dermal–epidermal junction (left) and larger nests of atypical melanocytes in the deeper dermis. Benign nevi have mutations in the *BRAF* gene that renders the MAP kinase pathway constitutively active to drive proliferation, presumably followed by oncogene-induced senescence. The dysplastic nevi are associated with loss of function in the tumor suppressor genes, such as *CDKN2A* and the *PTEN* gene, to potentially bypass senescence. **C:** Early melanoma: in-situ (left) with a proliferation of large, atypical melanocytes along the dermal–epidermal junction extending as single cells into the upper layers of the epidermis and early invasive melanoma (right) with transformed melanocytes extending into the upper dermis as small nests and single cells. **D:** Advanced VGP invasive melanoma is shown with large numbers of transformed cells extending deep into the dermis and subcutaneous tissue. Alterations in cells located in **C** and **D** are associated with changes in expression of integrins, cadherin, and molecules involved in metastases.



inducible pigmentation is tanning, which occurs in response to UV exposure, particularly UVA wavelengths.

Melanin includes eumelanin (the melanin of brown and black pigments) and pheomelanin (red pigment). Intermediate metabolites in melanin biosynthesis include highly reactive compounds, such as quinones, which end in cell damage or death. Generation of reactive products is prominent in the biosynthesis of pheomelanins, possibly contributing to the increased risk of melanoma in persons with red or fair hair and fair skin.

Genetic Determinants of Pigmentation

Skin, eye, and hair colors are determined by the type of melanin. Racial differences in skin pigmentation are not due to differences in the density of melanocytes but rather to how melanin is synthesized and packaged (10). In white skin, fewer and smaller melanosomes are produced in melanocytes, whereas in black skin, melanocytes produce larger and more numerous melanosomes, which in keratinocytes are distributed singly leading to greater absorption of photons. In the United States, blacks and Latinos

have a more than five- to tenfold reduced incidence of melanoma compared with whites.

In the context of melanoma, the most important genetic determinant of pigmentation is the receptor for melanocyte stimulate hormone (MSH), called MC1R. This is a transmembrane G protein–coupled receptor expressed by epidermal melanocytes that generates a cAMP second messenger through adenylate cyclase to regulate gene expression, including *MITF* (Figure 36-2; 11). MC1R is polymorphic in humans, and different allelic variants determine distinct skin phenotypes (12). MC1R variants that strongly transduce MSH signals lead to synthesis of darker pigment (eumelanin); in contrast, deficient MC1R signals are associated with synthesis of pheomelanin and red and other fair complexions.

One relevant group of loss-of-function MC1R alleles determines the Red Hair Color phenotype, characterized by red hair, fair complexion, difficulty in tanning, and the tendency to freckle, which are phenotypic characteristics of individuals with an approximate 1.2 to twofold increased risk of melanoma. Inheritance of Red Hair Color MC1R alleles is associated with

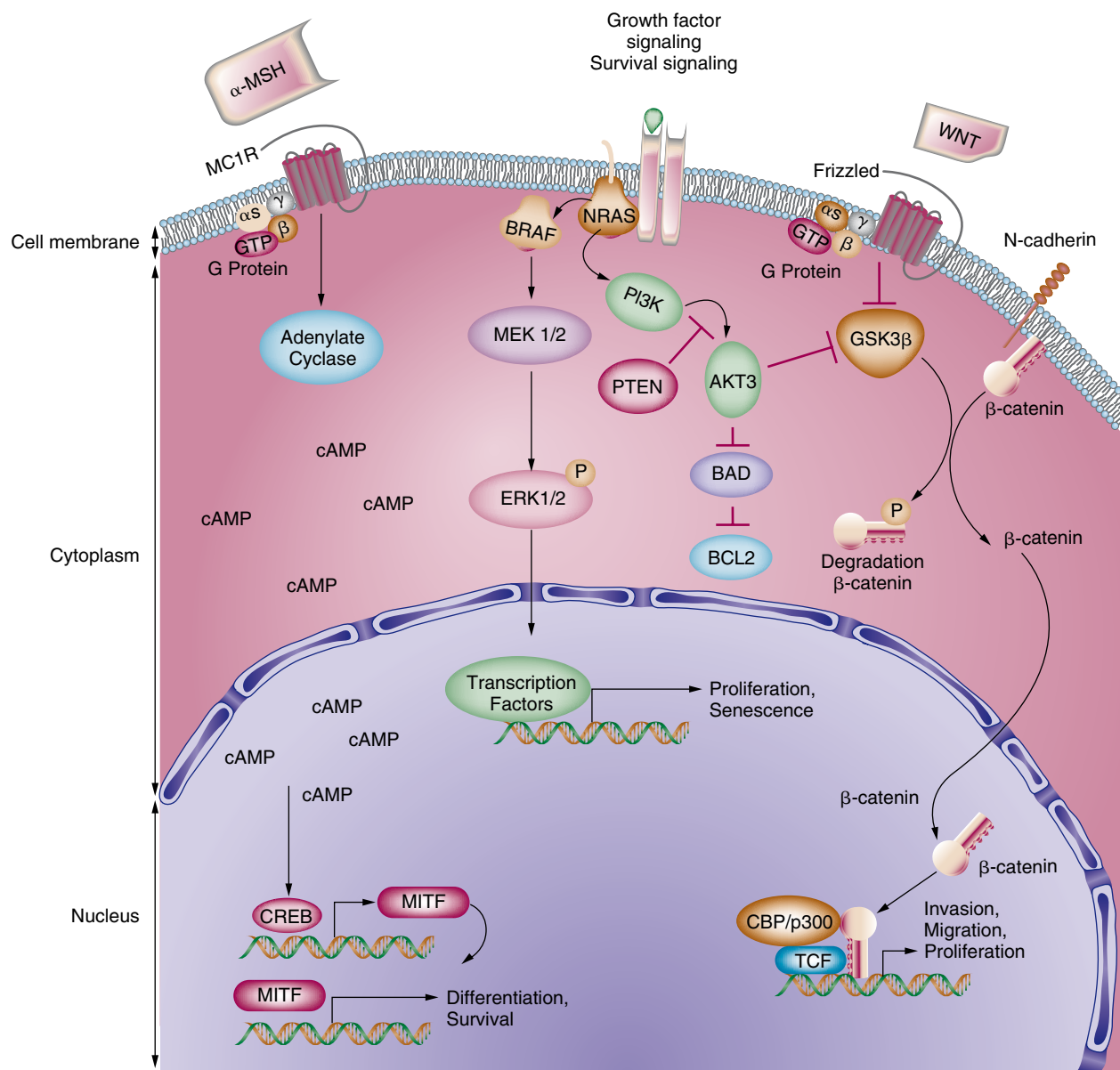


FIGURE 36-2 INVOLVEMENT OF RECEPTOR TYROSINE KINASE ACTIVATION, MC1R AND THE cAMP, MAP KINASE, AKT AND β -CATENIN PATHWAYS IN THE DEVELOPMENT OF MELANOMA. Melanocyte-stimulating hormone (MSH) is the ligand for the melanocortin receptor (MC1R). *MC1R* variants are a cause of red hair and fair skin. MC1R stimulates adenylyl cyclase, thereby activating cyclic AMP (cAMP) response element binding protein (CREB) and inducing MITF expression. Phosphorylated MITF in turn transactivates the expression of survival and differentiation genes. *MC1R* variants have been documented as a modifier of risk for melanoma development in individuals from kindreds prone to familial melanoma with *INK4*/*CDKN2* alterations. Growth factors and survival stimuli signal through cell surface receptors to activate mitogen-activated protein kinases (MAPKs), which induce cell cycle progression and proliferation. In general, adaptor proteins link the receptor to RAS (most importantly, NRAS for melanoma). Activated RAS triggers BRAF, which in turn induces phosphorylation of mitogen-activated protein kinase (MEK). MEK activates extracellular-related kinases (ERKs). ERK kinases translocate to the nucleus to activate transcription factors that, in turn, induce cell cycle progression. Melanoma is associated with mutations that render NRAS constitutively active, thus triggering the RAF-MEK-ERK pathway. BRAF mutations can also activate the RAF-MEK-ERK pathway. NRAS can also activate AKT through PI3K activation. Receptor signaling can activate PI3K, which in turn activates AKT to affect the expression of proteins involved in cell cycle progression, growth and survival. PTEN attenuates this pathway by blocking the activation of AKT. The PI3K-Akt pathway may be activated through loss or mutation of the inhibitory tumor suppressor gene PTEN or through gene amplification of AKT3. The WNT protein binds to its receptor frizzled (a G-protein-coupled receptor) and induces the inactivation of GSK3 β , a kinase that phosphorylates β -catenin to target it for degradation by the proteasome. Unmodified β -catenin accumulates in the cytoplasm and translocates to the nucleus.

Cadherins participate in the maintenance of proper cell-cell contacts. The intracellular domain of cadherins contacts the actin cytoskeleton and influences intracellular signaling. They are associated with large protein complexes including β -catenin. Modification of β -catenin leads to its translocation to the nucleus to associate with the CBP/p300-TCF transcription complex, also known as LEF/TCF. These factors transactivate the expression of invasion, migration and proliferation genes. In melanoma, decreased E-cadherin expression and ectopic N-cadherin expression increases cell survival and invasion by stimulating β -catenin signaling. Figure 36-2 is based in part on figures from ScienceSlides, VisiScience Corp.

increased risk of melanoma, even in individuals with darker skin. These observations implicate factors in addition to skin color and UV absorbance, such as generation of mutagenic molecules during inflammation and/or pigment synthesis following

sun exposure. Even inheritance of a single Red Hair Color MC1R allele is associated with decreased capacity to respond to cutaneous damage by UV radiation, potentially contributing to melanoma risk.

Melanoma Progression

Common Benign Nevus and Dysplastic Nevus

There is no common agreement on what are the exact precursor lesions for melanoma. When benign melanocytes are clustered in nests in the epidermis and/or the dermis (Figure 36-1), the cutaneous lesion is designated a common nevus (13). Typically, these lesions are pigmented, smaller than 6 mm in diameter, flat or slightly raised, and have uniform color. Although individual common nevi are not considered precursors for melanoma, the density of nevi on a person is one of the most important risk factors for developing melanoma (14,15). This may reflect a simple quantitative increase in melanocytes or may involve genetic or biologic differences in melanocytes in nevi.

The dysplastic nevus or atypical mole is a precursor lesion for melanoma; both are larger than 6 mm in diameter with irregular and asymmetric borders and variability in color. The frequency of malignant transformation of an individual dysplastic nevus to melanoma is low, but individuals with a single dysplastic nevus may have a twofold increased risk for melanoma compared with the general population, making dysplastic nevi a marker for increased risk. A 12-fold increase has been reported with 10 dysplastic nevi (15). The most compelling argument has come from patients with the dysplastic nevus syndrome (DNS), originally reported as the atypical mole syndrome, characterized by large numbers of dysplastic nevi with evidence of familial inheritance. These individuals have relative melanoma risks more than 1,000-fold, and lifetime risks approaching 100% when two family members have a history of melanoma (16). In several cases, DNS patients have been found to harbor germ-line mutations in the tumor suppressor gene *INK4A* (17,18). However, most DNS patients do not harbor *INK4A* mutations, and additional genes contribute to the DNS phenotype.

Pathologic Classification of Primary Melanoma

The single most important prognostic variable is predicting metastasis and survival in primary melanoma of the skin is the depth of invasion (13). A distinction between radial growth phase (RGP) and vertical growth phase (VGP) has been made (19). The RGP is characterized by horizontal spreading of transformed melanocytes along the plane of the epidermis, whereas the VGP is characterized by invasion of melanoma cells perpendicularly into the underlying skin (Figure 36-1). Pure RGP melanoma rarely metastasizes, while the depth of the VGP into the skin predicts risk of metastases.

Two systems are used by pathologists to describe the depth of invasion into the skin (Figure 36-1; 13). The original Clark's system (20) reflects the anatomic location of the lower edge of the lesion in the skin. The more accurate Breslow system quantitates the depth of invasion into the skin from the epidermis (21).

Staging of Melanoma

In stages I and II, Breslow thickness is the strongest predictor of survival. Most patients presenting with stage I disease, tumors of 1.0 mm deep, will never develop metastases. As the depth of invasion increases (stage II), the risk of metastases steadily grows. In stage III melanoma, where metastases are confined to tissues in the region of the primary lesion, in most cases lymph nodes, the most important prognostic variable is the number of involved nodes. The 5-year survival rate ranges from lower than 25% to approximately 70%, depending on the size and number of the metastases. Stage IV melanoma is defined by distant metastases. Five-year survival for stage IV disease is 10%, with a median survival of 6 to 12 months.

Epidemiology

The incidence of melanoma continues to rise worldwide, but this rise is most prominent in Western countries and Australia. In the United States, the lifetime risk of developing melanoma is estimated to be 1 in 75 for individuals born in 2005. Melanoma is the sixth most common cancer in the United States, the second most common cancer in women aged 30 to 34, and the most common cause of cancer death in young men ages of 25 to 34, leading to a relatively high impact in terms of actuarial years of life lost. Although an increasing proportion of melanomas are diagnosed at an early, curable stage, the death rate for melanoma continues to rise slowly and steadily. Melanoma tends to occur at a relatively early age compared with most other solid tumors, with a median age range of 50 to 55 years, suggesting that a relatively small number of cellular and genetic events are required for transformation to melanoma.

Sun Exposure

The primary determinants for melanoma risk are genetic, particularly skin type (e.g., MC1R genotype), the density of nevi, and familial predisposition (e.g., *INK4A/CDKN2A* mutations or loss), which can be modified by sun exposure. The clearest environmental risk factor is exposure to sunlight, although not a strong risk factor. Intermittent, high-intensity exposure to sunlight has been proposed to play a role in promoting melanoma (22). The most compelling epidemiologic data implicating sun exposure come from studies in areas with substantial fair-skinned populations, showing that the relative risk of melanoma rises as one lives closer to the equator, which in turn relates to increasing doses of solar radiation at ground level. For instance, the highest incidence of melanoma in the world is in northern Australia, where a relatively fair-skinned population of Northern European descent lives close to the equator. In contrast melanoma rarely is diagnosed among the dark-skinned aboriginal Australian population. Individuals with the greatest risk for developing sun-induced melanoma are those affected by the disease xeroderma pigmentosum. These patients have genetic defects in their ability to repair UV-damaged DNA and have a more than 1,000-fold increased risk of developing melanoma compared with the normal population (23). Although this is an exceedingly rare disease, it does provide additional support for a role of UV light in the pathogenesis of melanoma.

Cell Surface Receptors and Signaling in Melanoma Cells

Growth Factors and Receptor Tyrosine Kinases

Melanoma cells are characterized by proliferation that is independent of exogenous growth factors (24). Growth and differentiation of healthy melanocytes seem to require signals from two different receptor tyrosine kinases (RTKs). In contrast, signaling through a single RTK only produces a short-lived burst in mitogen-activated protein (MAP) kinase activity, insufficient to sustain proliferation. Relevant receptors include basic fibroblast growth-factor (bFGF) receptor and the KIT receptor. In the epidermis, bFGF is produced by keratinocytes, and production is increased sixfold in response to UV irradiation (25). Interestingly, one of the key early characteristics during melanocyte transformation is the ability to produce growth factors, including bFGF, to form autocrine stimulatory loops (25). This leads to low-level stimulation of the MAP kinase pathway to promote proliferation without differentiation.

Survival and migration of melanocytes during migration from the neural crest to the skin depend, at least in part, on the RTK *c-KIT* and its receptor KIT ligand/stem cell factor. Activation mutations in KIT have been implicated in gastrointestinal stromal tumors and several other rare tumor types. The KIT receptor is expressed by epidermal melanocytes in nevi and by melanoma cells in the basal layer of the epidermis. Moreover constitutive KIT activation induces melanocyte transformation (26). However, KIT expression is lost in the dermal component of nevi and by melanoma cells invading into the dermis. In fact, apoptosis can be induced in melanoma cells lacking KIT. Therefore, the KIT receptor is a necessary component for melanocyte survival and for the maintenance of a differentiated state. However, loss of signaling through this receptor seems to be required as an early step in melanomagenesis, at least in a subset of melanomas. Interestingly, the ability of neural crest cells to migrate and survive as they traffic through different tissues to the skin to form melanocytes depends on a functional KIT receptor; but paradoxically at least some melanomas disable this same system.

Amplifications and oncogenic mutations in KIT have been discovered in a subset of melanomas arising at nonexposed sites (mucous membranes, acral skin; e.g., soles, palms, and nail bed) and in skin with chronic sun damage. These findings implicate the KIT oncogene in the pathogenesis of melanoma subtypes not linked to sun exposure and to melanoma linked to chronic sun exposure; this is in sharp contrast with the more prevalent types of melanoma linked to intermittent sun exposure where oncogenic mutations in the RAS pathway are implicated.

Oncogenic NRAS and BRAF and the MAP Kinase Pathway

Activating mutations in NRAS or its downstream effector molecule BRAF to drive the MAP kinase pathway are present most

melanomas, specifically those arising in cutaneous sites of intermittent sun exposure (Figure 36-2; 27), stimulating proliferation. Gain-of-function mutations of NRAS and BRAF are reported in $\approx 10\%$ to 15% and $\approx 50\%$ to 70% of melanoma cases, respectively. These mutated gene products are almost never expressed together because an activation mutation in NRAS will constitutively drive BRAF to stimulate proliferation. BRAF or NRAS deficiency or pharmacologic inhibition of the downstream MAP kinase effector molecule MEK in human melanoma cells with these mutations suppresses growth and tumorigenicity, pointing to the potential importance of this pathway for maintenance of the transformed phenotype, and suggesting a form of “oncogene-addiction” for melanoma cells.

Remarkably, BRAF mutations occur at a similar frequency in benign nevi, reported around 50% to 60% (28). Given the presence of activating mutations of BRAF in nevi, which are benign lesions that remain stable for many years or decades, other genetic events must be required to further transform these lesions. A clue has come from studies in normal human melanocytes, which have revealed that mutant BRAF promotes cell senescence through a process called oncogene-induced senescence by inducing the expression of the cell cycle inhibitor INK4A, one of the products of the CDK2NA locus, which is discussed in more detail in Tumor-Suppressor Genes (29). The growth arrest can be overcome by mutations in the INK4A gene, which lead to deficient expression or activity of its protein product INK4A. Mutations in INK4A are frequent in melanoma and have been the most frequent genetic events implicated in familial melanoma. Therefore, activated mutant BRAF and deficient INK4A cooperate, with BRAF driving senescence or, in the context of INK4A deficiency, uncontrolled cell proliferation.

The Phosphatidylinositol-3-Kinase Pathway

NRAS signals multiple downstream effectors through activation of the RAS-MAP kinase and the phosphatidylinositol-3-kinase (PI3K)-AKT pathways, the latter promoting cell cycle progression and inhibiting apoptosis (Figure 36-2). In melanoma, the PI3K pathway is also activated by deficiency in the tumor suppressor PTEN or by AKT3 amplification. AKT phosphorylation results from increased levels of the lipid phosphatidyl inositol phosphate (PIP3). PTEN, a dual-specificity phosphatase, regulates extracellular growth signals, which use PIP3 as an intracellular second messenger.

The involvement of the PI3K-AKT pathway in melanoma is evidenced by allelic loss of PTEN in $\approx 20\%$ of tumors (30). The detection of both PTEN inactivation and BRAF mutations in melanoma has further suggested a cooperative effect of these two pathways. Moreover, the selective activation of AKT3 has been reported in $\approx 40\%$ to 60% of sporadic melanoma. AKT3 activity increases during melanoma tumor progression, with highest levels in advanced metastases. AKT3 deregulation occurs through a combination of overexpression, increased gene copy number, and decreased PTEN protein levels through loss or haploinsufficiency of the PTEN gene.

Tumor-Suppressor Genes

Analyses of melanoma prone families led to identification of the CDKN2A locus on chromosome 9p21 as a susceptibility gene in

familial melanoma (24). Loss of heterozygosity and mutations in *CDKN2A* cosegregate with melanoma susceptibility in melanoma kindreds, with germ-line lesions identified in 25% to 40% of familial melanoma-prone families (18). Moreover, mice with inactivation at the *CDKN2A* locus are prone to chemically induced melanoma. Subsequent studies have revealed similar frequencies of somatic *CDKN2A* mutations in sporadic melanoma.

Remarkably, the *CDKN2A* locus encodes two distinct tumor suppressor gene products, INK4A (p16^{INK4a}) and ARF (ARF for alternate-reading frame; p14^{ARF} in humans, p19^{Arf} in mice), which are each transcribed via alternative promoters and first exons (Figure 36-3; 24). The second exon is shared and translated in two different reading frames, leading to expression of INK4A and ARF products with no homology. The INK4A protein sequesters cyclin-dependent kinase 4 (CDK4) and CDK6, thereby preventing phosphorylation of the RB protein (to generate inactive pRB) by CDK4 and CDK6 (Figure 36-3). RB in its active state (hypo-phosphorylated) in turn sequesters the transcription

factor E2F to inhibit cell cycle progression, leading to uncontrolled cell cycle progression (31). Mutations in the *CDKN2A* locus can inactivate *INK4A*, *ARF* or both genes.

The ARF protein stabilizes p53 by preventing HMD2-mediated degradation; HMD2 normally triggers the ubiquitination of p53 for proteasome degradation (24). Accumulation of p53 leads to cell cycle arrest at the G₂-M phase of the cell cycle, leading to a DNA damage repair response or apoptosis to protect the cell from transformation. An absence of ARF inactivates p53, favoring malignant transformation. Although p53 is mutated in many common cancers, p53 mutations have not been found with any measurable frequency in melanoma. However, p53 protein expression increases with the depth of tumor invasion, suggesting that other mechanisms can increase p53 expression in melanocytic lesions. Mutated ARF gene products provide one explanation for the low frequency of p53 mutations in melanoma.

Less frequently, the RB pathway has been inactivated by mutations in the *CDK4*, the downstream target of INK4A both

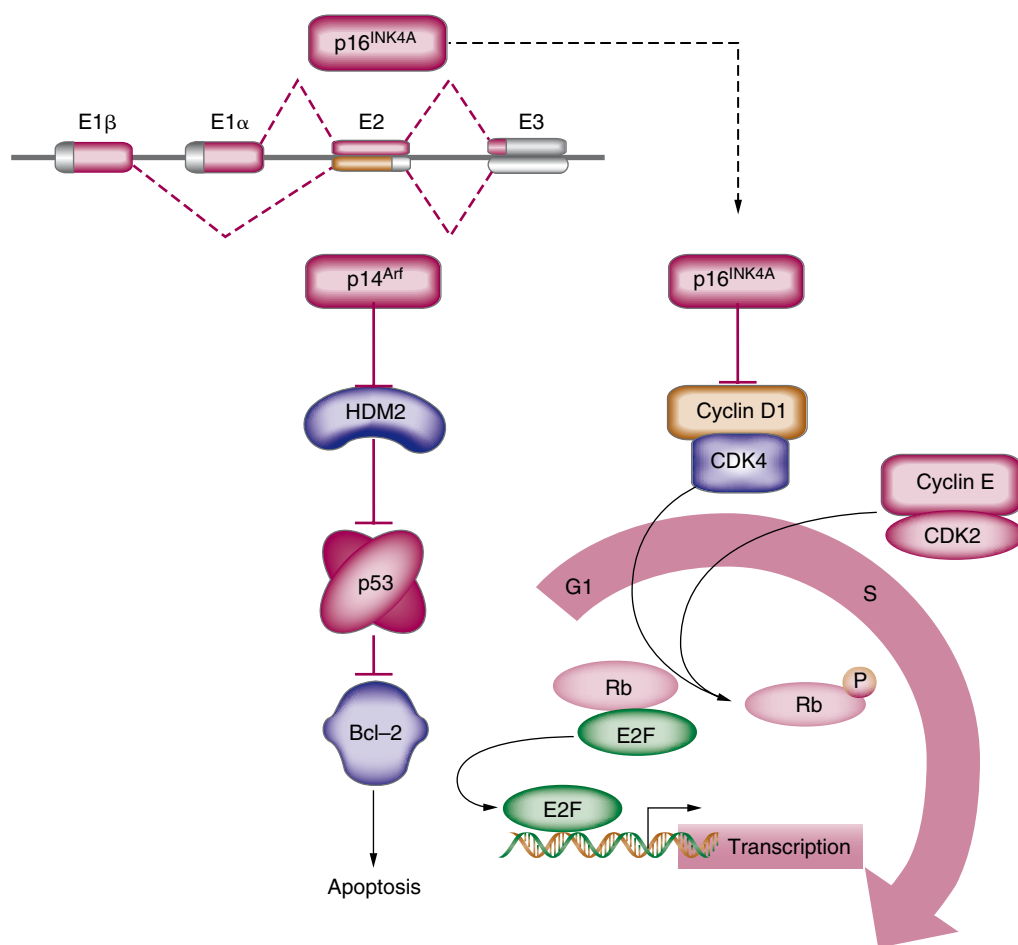


FIGURE 36-3 CDKN2A LOCUS AND MECHANISM OF ACTION OF P14/ARF AND P16/INK4 GENES IN MELANOMA. The *CDKN2A* locus is composed of four exons. It encodes two tumor-suppressor genes, *ARF* (p14^{ARF}, p19^{Arf} in mice) and *INK4A* (p16^{INK4a}) via alternative reading frames. p14^{ARF} is encoded by exons E1α, E2, and E3, and p16^{INK4a} is encoded by exons E1β, E2, and E3. The p16^{INK4a} protein sequesters CDK4, maintaining RB in an active hypophosphorylated state. CDKs regulate progression through the cell cycle and CDK inhibitors oppose this action. Cyclin D1 associates with CDK4 to control the early G₁ to S cell cycle phase transition, and cyclin E and CDK2 control late G₁ to S cell cycle phase transition. Deficiency of or a mutation in *INK4A* leads to binding of CDK4 to cyclin D1 and phosphorylates RB. Phosphorylated RB releases the E2F transcription factor from the E2F-RB complex. E2F subsequently leads to the expression of the E2F target genes, which in turn promote the G₁ to S cell cycle phase transition. The tumor suppressor p14^{ARF} sequesters the ubiquitin ligase HDM2 from a complex coupling HDM2 and p53, thereby increasing p53 activity. Active p53 triggers DNA repair and cell cycle arrest or alternatively promotes apoptosis. p53-BCL2 binding is associated with decreased BCL2-BAX interactions and increased apoptosis. In the absence of p14^{ARF}, HDM2 complexes with p53 and impairs mechanisms that normally target genetically damaged cells for cell cycle arrest and apoptosis. Loss of the *CDKN2A* targets both the p53 and RB pathways. (From ScienceSlides, VisiScience Corp., with permission.)

in sporadic and familial melanoma. CDK4 is rendered constitutively active with a mutation, preventing the binding of CDK4 to INK4A. Mice with this mutation are prone to chemically induced melanoma. Mutations in *CDK4* and *INK4A* are predictably mutually exclusive. Moreover, patients with mutations in the *RB* gene are predisposed to melanoma; patients cured of retinoblastoma have an 80-fold increased risk for melanoma.

Genes Involved in Invasion and Metastases

The body of work described in the preceding section has directly implicated receptors and downstream molecules in the MAP kinase–RB pathway, and indirectly implicated the PI3K–AKT pathway, in melanoma pathogenesis (24). Genes involved in later stages of melanoma progression, particularly metastases, are not well characterized. *MITF*, encoding the basic helix-loop-helix leucine zipper transcription factor, which is a master regulator of development and differentiation of melanocytes (see Melanocyte Development and Melanocytes Stem Cells), is amplified in some melanomas, particularly metastases. *BRAF* activating mutations and *INK4A* inactivation have been associated with *MITF* amplification, and ectopic *MITF* expression in conjunction with activating *BRAF* mutations transform primary human melanocytes, revealing that *MITF* can function as a melanoma oncogene (32). These findings have suggested a lineage-dependence (or “lineage addiction”) as a mechanism to drive oncogenesis (33).

Studies using comparative genomics in mice and humans have identified NEDD9, a signaling adaptor protein, as a gene implicated in metastases. The role of NEDD9 is to enhance invasion and metastasis, possibly through regulation of focal adhesion kinases (24).

As the depth of invasion of primary melanoma increases, the frequency of metastases increases (Figure 36-1). A characteristic of melanoma progression is that the melanocytic cell, which in a healthy condition is constantly responding to its environment to down-regulate growth and promote differentiation, transforms to a cell that proliferates autonomously, resists differentiation and growth inhibition signals, invades surrounding tissues, and directs the surrounding stroma to supply relevant growth factors and a blood supply (34).

A requisite step in the early invasive process is breakdown of the basement membrane and extracellular matrix in the skin. Invasive melanomas secrete proteolytic enzymes, including matrix metalloproteinases (MMPs), serine proteases, and other enzymes such as hyaluronidase and heparanase. MMP2, which is expressed at low levels in benign nevi, is increased in primary and metastatic melanomas. In addition, melanoma cells stimulate surrounding fibroblasts to secrete proteoglycans, which result in a stroma more conducive to the migration of tumor cells and to angiogenesis (34). These released extracellular matrix proteins bind growth factors, including bFGF; degradation of the extracellular matrix liberates these factors, which are not only mitogens for melanoma cells but also are angiogenic stimuli for endothelial cells (34). Melanoma cells themselves produce a number of potentially pro-angiogenic factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor, bFGF, pleiotrophin, transforming growth factor- α , and interleukin-8.

The expression of integrins in melanomas is implicated in adhesion to extracellular matrix, promoting motility, invasion and metastasis, and signaling through the MAP kinase and protein kinase C pathways. Tumor progression correlates with decreased expression of $\alpha_6\beta_1$ integrin, and with increased expression of the integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$. Treatment of melanoma cells with antibodies to $\alpha_4\beta_1$ and other integrins prevents metastases, and this approach is being explored in clinical trials for patients with melanoma.

Cadherins are multifunctional transmembrane proteins that are crucial for the maintenance of proper cell–cell contacts during development, acting both as receptors and ligands. The intracellular domain of cadherins contacts the actin cytoskeleton and influences intracellular signaling through association with large protein complexes, particularly β -catenin (Figure 36-2). E-cadherin expression maintains contact between melanocytes and keratinocytes in the epidermis, inhibiting melanocyte proliferation and maintaining a differentiated dendritic morphology. Loss of E-cadherin and de novo N-cadherin expression together have been implicated in melanoma progression and metastasis, with progression of primary melanoma from RGP to the VGP characterized by the loss of E-cadherin expression and up-regulation of N-cadherin. Moreover, N-cadherin expression increases melanoma cell survival by stimulating β -catenin signaling, leading to translocation of β -catenin to the nucleus where it binds to LEF/TCF transcription factors, activating genes in *MITF* and *CCND1* (the latter encoding the cell cycle promoter cyclin D1; Figure 36-2). Expression of β -catenin is regulated by the WNT pathway, an important player in neural crest development, and by AKT, through inhibition of GSK3 β kinase, which normally phosphorylates β -catenin to trigger its destruction by the proteasome (Figure 36-2).

Future Directions

Advances in genetics and the sequencing of the human genome are leading to a comprehensive understanding of the molecular pathways in specific types of cancer. One of the consequences is the realization that individual cancer types need to be further classified into heterogeneous subtypes with distinct molecular pathways. These characteristics will be important for diagnosis, prediction of disease progression, and identification of new genes involved in pathogenesis. In the future, these classifications are likely to be used to guide therapy for individual patients through the selection of specific therapeutic agents that target specific signaling pathways.

Genomic profiling and transcript analysis of melanoma have identified distinct, previously unrecognized subclasses of melanoma. Most melanomas arising in intermittently sun-exposed skin, without chronic sun-induced damage, have mutations in *BRAF* or *NRAS* (35). On the other hand, other melanoma subtypes rarely have activating mutations in *BRAF* or *NRAS* and are characterized by frequent amplification of genes encoding proteins downstream of MAP kinase, specifically CDK4 and the related cyclin D1 (*CCND1*; Figure 36-3). Therefore, *CDK4* and *CCND1* are likely oncogenes in melanomas independent of *BRAF* or *NRAS* mutations. Furthermore, subtypes with amplifications

and oncogenic mutations in *KIT* are prevalent in melanomas arising in acral, mucosal sites and chronically sun-exposed locations. Genomic profiling can distinguish different subtypes with some accuracy; for example, acral melanoma can be distinguished from mucosal melanoma with 89% accuracy by genomic profiling (35). Thus, these observations show that there are distinct genetic pathways in the development of different forms of melanoma, with implications for prevention, diagnosis, and treatment.

A number of small molecules that target KIT, RAS, BRAF, and downstream MAP kinase effector molecules, as well as PI3K, AKT, and their downstream effectors, are being developed to test in clinical trials. There is substantial excitement in the oncology community about these targets. However, a great deal of clinical research needs to be done through carefully designed clinical trials to validate

these drugs and targets. Likely, drugs will need to be combined to target multiple pathways (e.g., BRAF-MAP kinase and PI3K pathways), perhaps with more standard cytotoxic agents.

Acknowledgments

We are grateful for expert editorial assistance from Dr. Stephanie Terzulli. The authors would also like to thank Tony Riley, Medical Graphics and Photography Department of Memorial Sloan Kettering Cancer Center, for his help with the figures. T.M. and A.N.H. are supported by Rita Annenberg Hazen Foundation, Swim Across America, and grants CA33049 and CA56821 from the National Cancer Institute to study melanoma.

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Soft-Tissue Sarcomas

Soft-tissue sarcomas are a heterogeneous group of neoplasms arising in mesenchymal tissue. There are more than 35 histologic subtypes, often associated with distinctive clinicopathologic features. Based on advances in our knowledge of the molecular pathogenesis of these tumors over the past decade, sarcomas can be subdivided into those with recurrent, usually quite simple genetic alterations (approximately one third of sarcomas; [Table 37-1](#)), which occur mainly in younger patients and those with nonrecurrent genetic alterations, the majority of which occur in older adults ([1](#)). Examples of the latter group include leiomyosarcomas, malignant peripheral nerve sheath tumors, and the heterogeneous group of high-grade pleomorphic and spindle cell sarcomas (often formerly known as so-called malignant fibrous histiocytomas), all of which lack specific recurrent genetic aberrations and which instead exhibit complex karyotypes including multiple chromosomal deletions, losses, and gains. These latter sarcomas frequently have alterations in the *p53* tumor suppressor pathway and a clinically aggressive course ([2](#)). Recurrent translocations in the former group are typically the only cytogenetic alteration present and often involve transcription factors (e.g., *WT1*, *PAX3*, *TFE3*, *FLI1*). This chapter focuses on three tumors with known genetic alterations, synovial sarcoma, well-differentiated liposarcoma, and gastrointestinal stromal tumor, as prototypical examples of translocation-associated, gene amplification-associated, and oncogenic mutation-associated sarcomas, respectively.

Synovial Sarcoma

Clinical Description and Pathology

Synovial sarcoma (SS) accounts for approximately 8% to 10% of all sarcomas; it is however the most common sarcoma of young adults ([3](#)). The peak incidence is between ages 15 and 40 and most occur before age 50 ([4](#)). Males are slightly more affected than females. SS most commonly arises in the extremities (90%), although almost any other site can be affected, including the head and neck, mediastinum, lung, retroperitoneum, abdominal wall, and gastrointestinal tract. It typically presents as a slowly growing, painful mass, with calcifications often being present radiologically ([5](#)). It is an

aggressive sarcoma, with a 5-year mortality rate ranging from 25% to 76% ([6–8](#)). Up to 50% of SS cases recur, usually within 2 years of initial diagnosis. The most common location for metastases is the lungs and the most important predictor of metastases is tumor size, with a size greater than 5 cm correlating with a significantly increased metastatic risk ([7](#)). Advanced age and advanced stage at presentation, as well as poorly differentiated histology, also correlate with a worse outcome. The long-term prognosis for SS is relatively poor, with a 10-year survival rate of 50% ([7](#)).

Histologically, SS is a mesenchymal neoplasm displaying varying degrees of epithelial differentiation and bearing no biologic relationship to synovial tissue. The tumor was originally designated SS because its tendency to occur near articular surfaces and its epithelioid component initially suggested origin from synovial tissue. However, later ultrastructural and immunohistochemical analyses have conclusively demonstrated true epithelial and not synovial differentiation ([3](#)).

SS is divided into two morphologic subtypes, biphasic and monophasic, on the basis of presence or absence of glandular epithelial differentiation. The biphasic variant is characterized by spindle cell areas intermingled with an epithelial component, often forming glands, tubules or nests ([Figure 37-1A](#)). The epithelial cells are usually larger, with paler nuclei and more abundant cytoplasm than the spindle cell component. The epithelial component can be very focal, making it difficult to detect unless highlighted by keratin stains. The spindle cell areas are composed of closely packed cells with hyperchromatic nuclei and scant cytoplasm, imparting an overlapping appearance, growing in sheets and/or fascicles. Keratins and other epithelial immunohistochemical markers are more abundant in the epithelial component; however, they (especially epithelial membrane antigen) are also expressed, albeit to a lesser extent, in the spindle cell areas. The monophasic variant is composed entirely of the spindle cell component and is the more common subtype ([Figure 37-1B](#)). Another characteristic hallmark of both biphasic and monophasic variants is the presence of branching vessels, termed “hemangiopericytoma-like,” as is wiry stromal collagen. SS cases with areas of densely packed small round to spindled cells and a high mitotic rate (with or without necrosis) are designated poorly differentiated, and as mentioned previously, have a worse prognosis.

Table 37-1 Soft-Tissue Sarcomas with Recurrent Genetic Alterations

Tumor	Recurrent Genetic Abnormality	Genes Involved
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(p36;q14)	<i>PAX3-FOXO1A</i> <i>PAX7-FOXO1A</i>
Alveolar soft-part sarcoma	t(X;17)(p11;q25)	<i>TFE3-ASPL</i>
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	<i>CREB1-EWSR1</i> <i>ATF1-FUS</i>
Clear cell sarcoma	t(12;22)(q13;q12)	<i>ATF1-EWSR1</i> <i>CREB1-EWSR1</i>
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	<i>PDGFB-COL1A1</i>
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	<i>WT1-EWSR1</i>
Embryonal rhabdomyosarcoma	LOH at 11p15	<i>BWSCR1A</i> <i>IGF2</i>
Ewings sarcoma/peripheral primitive neuroectodermal tumor	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)(q12;q12) t(2;22)(q33;q12)	<i>FLI-1-EWSR1</i> <i>ERG-EWSR1</i> <i>ETV1-EWSR1</i> <i>E1AF-EWSR1</i> <i>FEV-EWSR1</i>
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12) t(9;17)(q22;q11) t(9;15)(q22;q21)	<i>NR4A3-EWSR1</i> <i>NR4A3-RBP56</i> <i>NR4A3-TCF12</i>
Gastrointestinal stromal sarcoma	Activating mutations	<i>c-kit</i> <i>PDGFRA</i>
Infantile fibrosarcoma	t(12;15)(p13;q25)	<i>ETV6-NTRK3</i>
Low grade fibromyxoid sarcoma	t(7;16)(q33;p11) t(11;16)(p11;p11)	<i>CREB3L2-FUS</i> <i>CREB3L1-FUS</i>
Inflammatory myofibroblastic tumor	t(1;2)(q22;p23) t(2;19)(p23;p13) t(2;17)(p23;q23) t(2;2)(p23;q13)	<i>ALK-TPM3</i> <i>ALK-TPM4</i> <i>ALK-CLTC</i> <i>ALK-RANBP2</i>
Malignant rhabdoid tumor	deletion 22q11	<i>SMARCB1</i>
Myxoid liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	<i>DDIT3-FUS</i> <i>DDIT3-EWSR1</i>
Synovial sarcoma	t(X;18)(p11;q11)	<i>SSX1-SYT</i> <i>SSX2-SYT</i> <i>SSX4-SYT</i>
Well-differentiated liposarcoma/atypical lipomatous tumor	12q14–15 amplification	<i>MDM2</i> <i>CDK4</i> <i>SAS</i> <i>HMGA2</i>

Genetics and Molecular Pathogenesis

SS contains a specific translocation between chromosomes X and 18-t(X;18(p11.2;q11.2)(3). This translocation results in the fusion of the *SYT* gene on chromosome 18 with *SSX1* or *SSX2* (or rarely *SSX4*) on the X chromosome. The resulting fusion gene contains *SYT* gene minus the final eight C-terminal amino acids and the C-terminus of the *SSX* gene (Figure 37-1C).

SYT is widely expressed during early murine embryogenesis and in the adult (3). The gene encodes a protein with three

possible SH2- and one possible SH3-binding domains (likely protein–protein interaction domains), a novel N-terminal domain (termed an “SNH domain”) and a C-terminal domain rich in glutamine, proline, glycine and tyrosine (QPGY domain) similar to those found in the *EWSR1* gene and other transcriptional activators (Figure 37-1C). There is in vitro evidence that *SYT* can act as a transcriptional activator and *SYT* has been shown to bind to the *SWI/SNF* chromatin remodeling complex (9). *SYT* also interacts with the p300 nuclear pore protein, and this interaction appears to promote cell–cell adhesion (10). An *SYT* deletion mutant lacking the eight C-terminal amino acids (the most common *SYT* mutant found in the *SYT/SSX* fusion gene product) acts in a dominant negative fashion, preventing cell adhesion to an extracellular matrix (10). These data suggest that loss of contact inhibition of cell growth through the dominant negative activity of the *SYT/SSX* fusion gene product may play a role in SS tumorigenesis.

SSX1 and *SSX2* share significant homology (81% identity) to each other and belong to a gene family whose expression is predominantly restricted to germ cells and tumors (3). The *SSX* protein contains an N-terminal domain similar to the Kruppel-associated box (KRAB) domain found in several transcriptional repressors, and an acidic C-terminal region (*SSX-RD*), which appears to be a novel transcriptional repressor domain (11) required for colocalization with polycomb proteins, which are important for maintaining transcriptional repression from one cell cycle to the next (Figure 37-1C; 12).

The transforming capability of the *SYT-SSX1* fusion gene product has been demonstrated in vitro and in vivo (13). The N-terminal region of *SYT* (which binds to the *SWI/SNF* chromatin remodeling complex) is required for the transforming properties of *SYT-SSX1*, suggesting a role for transcriptional regulation by *SYT-SSX1* via the *SWI/SNF* complex in SS tumorigenesis. It is interesting to note that the *SYT-SSX* chimeric protein has both transcriptional activation (QPGY) and transcriptional repressor (*SSXR*D) domains, suggesting association with two opposed complexes. The requirement of the *SWI/SNF* binding region for transformation suggests that transcriptional activation may play the more important role in *SYT-SSX1* tumorigenesis; however this remains unverified.

Although SS invariably shows some evidence of epithelial differentiation, there is no sign of origin from (or continuity with) epithelium, and the tumor’s predominant line of differentiation and its normal cellular counterpart is unclear. The biochemical pathways important for SS development and growth remain largely uncharacterized. A study demonstrated significant up-regulation of insulin-like growth factor2 (*IGF2*) by *SYT-SSX* and a requirement for this protein in *SYT/SSX*-induced tumor formation in nude mice (14). However, the mechanism of *IGF2* regulation by *SYT-SSX* remains uncertain. Analysis of gene expression profiles induced by *SYT-SSX1* identified reduced expression of the tumor suppressor gene *DCC*, *XRCC4* (a DNA repair protein), and *MSH2* (a DNA mismatch repair gene) (15), although whether deregulation of these particular genes plays a role in SS development is unknown.

In the literature, it appears that biphasic SS much more commonly carry the *SYT-SSX1* fusion, whereas monophasic

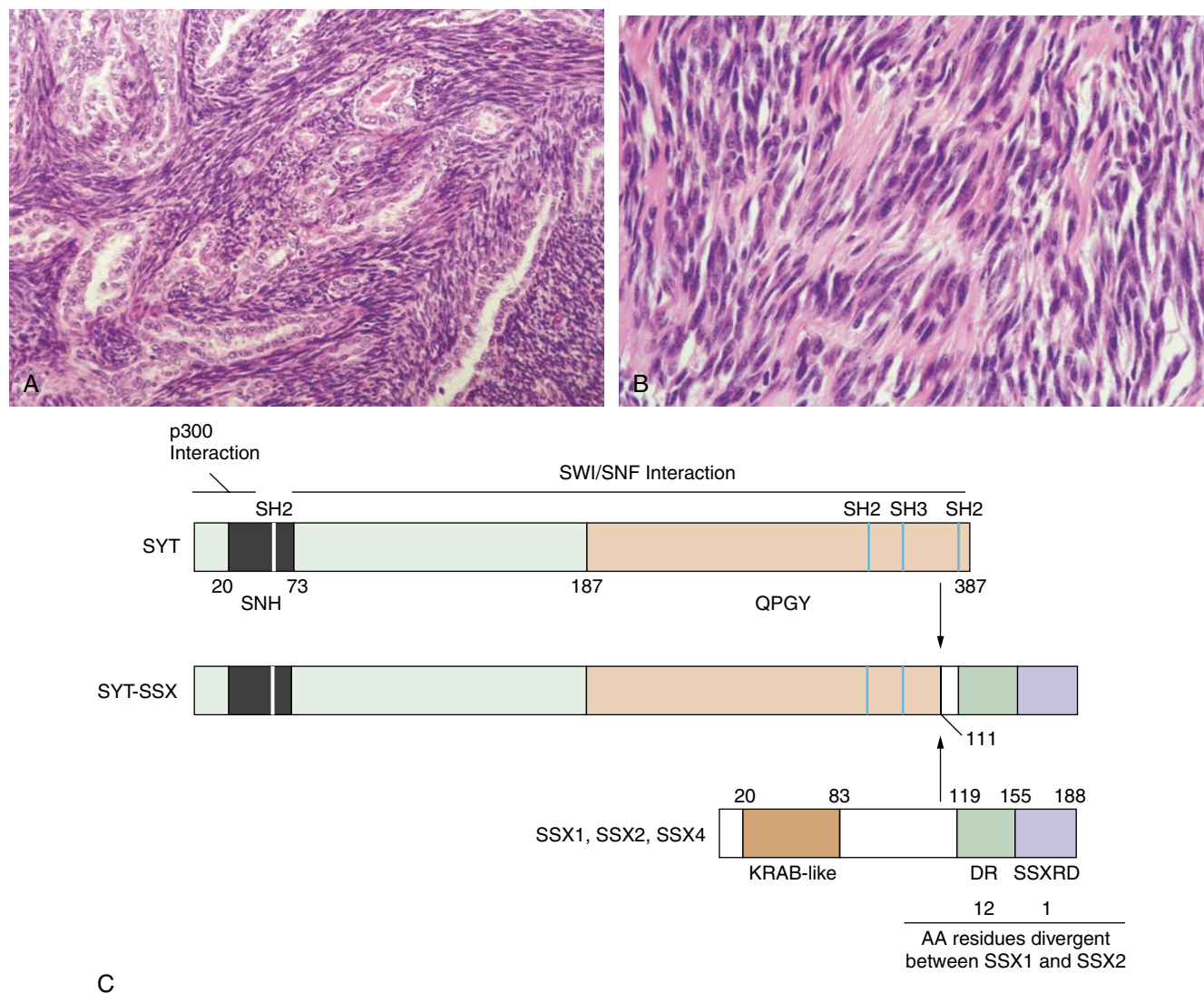


FIGURE 37-1 **SYNOVIAL SARCOMA.** **A:** Biphasic synovial sarcoma, showing glandular differentiation within a spindle cell background. **B:** Monophasic synovial sarcoma, demonstrating the typical densely packed spindle cells growing in a fascicular pattern associated with wiry stromal collagen. **C:** Diagram of SYT, SSX, and SYT-SSX fusion proteins. AA, amino acids; DR, SSX divergent region; KRAB, Kruppel-associated box; QPGY, SYT glutamine, proline, glycine, and tyrosine-rich domain; SNH, SYT N terminal domain; SSXRD, SSX repressor domain.

SS show either *SYT-SSX1* or *SYT-SSX2* (3,16,17). This trend between fusion gene product and histologic type suggests the possibility that the *SYT-SSX1* fusion gene is more efficient at promoting epithelial differentiation than the *SYT-SSX2* fusion gene. However, some biphasic SS carries the *SYT-SSX2* rather than the *SYT-SSX1* transcript, so this reported association may not be significant.

Attempts have also been made to correlate fusion type with prognosis, although the results are conflicting. Several studies have suggested that patients with the *SYT-SSX2* translocation have a better prognosis than those with the *SYT-SSX1* transcript (17,18); others have found no statistically significant correlation between fusion type and prognosis (16). All of the studies were retrospective in nature and none could control for differences in treatment or selection bias in follow-up data. The proposed correlation of fusion gene type with prognosis therefore remains to be confirmed.

Atypical Lipomatous Tumor/ Well-Differentiated Liposarcoma

Clinical Description and Pathology

Liposarcoma as a class is the most common malignant soft-tissue neoplasm. Of the several distinct subtypes, atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDL), a sarcoma of intermediate (locally aggressive) malignancy, is the most common (19). It is a tumor of older adults, most often presenting in the fifth to seventh decades. Males and females are equally affected. ALT/WDL occurs most frequently in the extremities or retroperitoneum, followed by the paratesticular region, mediastinum, and head and neck. They tend to be deep-seated, slowly growing masses, and thus are often quite large before coming to clinical attention.

Anatomic location is the most important prognostic factor, since ALT/WDL does not metastasize unless dedifferentiation has occurred (see following sections). Indeed, although the terms “atypical lipomatous tumor” and “well-differentiated liposarcoma” are synonymous, tumors in surgically resectable locations in the limbs and trunk are labeled atypical lipomatous tumors since wide excision is curative. In contrast, those located in the retroperitoneum and mediastinum, where wide excision is difficult, are referred to as well-differentiated liposarcoma, since repeated and uncontrolled local recurrences are very common, and mortality is high even in the absence of dedifferentiation or metastasis.

Histologically, ALT/WDL is divided into four subtypes (without prognostic implications): adipocytic (lipoma-like), sclerosing, inflammatory, and spindle cell, of which the first two are the most common (19). In general, ALT/WDL is composed of relatively mature adipose tissue with significant variation in cell size and varying degrees of nuclear atypia in adipocytes and stromal cells, particularly within fibrous septa. Lipoblasts are often present; however, they are frequently rare and are not required for the diagnosis. Sclerosing ALT/WDL is characterized by collagenous stroma containing pleomorphic hyperchromatic stromal cells, and is most common in the retroperitoneum and paratesticular region. Inflammatory ALT/WDL is rare but is important to recognize in that it can be misdiagnosed as Hodgkin lymphoma, other sarcomas, or even as a nonneoplastic process, due to the extensive chronic inflammation present. Spindle cell ALT/WDL is composed of relatively bland spindle cells admixed with adipocytes in a fibrous or myxoid stroma.

Dedifferentiation, defined by progression to nonlipogenic, often high-grade sarcoma, occurs in approximately 10% of ALT/WDL. It most commonly occurs in the retroperitoneum, since dedifferentiation appears to be a time-dependent (or size-dependent) phenomenon and retroperitoneal ALT/WDL often remains asymptomatic until it reaches a large size and tends to have a protracted clinical course due to its relative unresectability. Histologically, dedifferentiated liposarcoma (DDLPS) is characterized in most cases by an abrupt transition from ALT/WDL areas to variably pleomorphic spindle cell sarcomatous areas. DDLPS has a 40% to 50% local recurrence rate, a 15% metastatic rate, and a 30% 5-year mortality rate (20).

Genetics and Molecular Pathogenesis

Giant marker and supernumerary ring chromosomes are the hallmark of ALT/WDL and are also present in DDLPS (21). These giant ring and marker chromosomes contain massive amplification of the 12q13–15 chromosomal region (22). A number of other chromosomal regions, including 12q21–22 and 1q21–25, have also been shown to be co-amplified. The *p53* regulator *MDM2*, located in 12q14–15, is consistently amplified in ALT/WDL, typically in association with neighboring genes, including *CDK4*, *SAS*, and *HMG2*. *MDM2* negatively regulates the tumor suppressor *p53* by targeting it for ubiquitin-mediated destruction (23). Approximately 30% to 40% of sarcomas in general display *MDM2* overamplification and 38% of tumors in mice overexpressing *MDM2* are sarcomas (24). However, a definitive requirement

for *MDM2* amplification (or any of the genes co-amplified with *MDM2*) in ALT/WDL tumorigenesis has not been established. Nutlins, a class of small-molecule inhibitors of *MDM2*, have been identified (25), and it will be interesting to see whether these compounds are effective in ALT/WDL and/or DDLPS treatment.

The molecular pathogenesis of tumor progression from ALT/WDL to DDLPS is also largely uncharacterized. DDLPS differ cytogenetically from ALT/WDL in that they often show additional complex karyotypic changes (21). DDLPS also have been shown to possess a greater degree of 12q amplification than ALT/WDL (26). However, whether these changes are important for tumor progression to dedifferentiation is unclear. Despite its histology, DDLPS has a less aggressive clinical course than most high-grade pleomorphic sarcomas in adults. The latter tumors typically display both *MDM2* and *p53* alterations which correlates with a poor prognosis (27). In contrast, although there are conflicting data regarding the frequency of *p53* mutation in DDLPS (28–30), it is likely mutated in only a minority of DDLPS.

Gastrointestinal Stromal Tumor

Clinical Description and Pathology

Gastrointestinal stromal tumors (GISTs) are the most common sarcomas of the gastrointestinal tract (31). The overall age range at presentation is broad; however, most GIST tumors are diagnosed in patients older than 50. GISTs occur with equal incidence in both males and females and can occur at any location in the gastrointestinal tract. The most common site is the stomach (50%), followed by the small intestine (25%), large intestine (10%), esophagus (5%), and rarely, the gallbladder, appendix, or pancreas. GIST can also arise at sites outside the tubular gastrointestinal tract, including the retroperitoneum, pelvis, mesentery, and omentum, although these extragastrointestinal GISTs account for only about 10% of all GISTs. Clinical presentation includes anemia secondary to gastrointestinal bleeding, early satiety, and intestinal obstruction; however, GISTs are also occasionally identified as incidental findings.

The most common metastatic sites for GIST are intra-abdominal, namely the liver, peritoneum, omentum, and mesentery. GISTs rarely spread to lymph nodes or to extra-abdominal sites and when they do, it tends to be late in the course of disease. The most important predictors of metastasis are tumor size and mitotic index, with a size less than 5 cm and a mitotic index less than 5 per 50 high power fields (HPFs) conferring a low risk of aggressive behavior and a size greater than 10 cm and/or a mitotic index greater than 10 per 50 HPF conferring a high risk of aggressive behavior (32). Occasionally however, even small GISTs (<2 cm) with a low proliferative rate can behave aggressively, thus follow-up is recommended even for those lesions with a low relative risk of metastasis.

Based on cytomorphology, GIST can be divided histologically into three categories, spindle cell, epithelioid, and mixed epithelioid and spindle cell type. Epithelioid areas are composed of cells growing in sheets or nests, with round nuclei and fairly

abundant eosinophilic cytoplasm (Figure 37-2A). The spindled cells are typically monomorphic, with vesicular chromatin, pale eosinophilic, almost syncytial cytoplasm, growing in short fascicles (Figure 37-2B). Pleomorphism is rare. Additional characteristic features of both spindle cell and epithelioid GIST are the presence of perinuclear vacuoles and the fibrillary nature of the cytoplasm.

GISTs frequently stain positively for smooth muscle markers (at least 30%–40% are positive for smooth muscle actin or caldesmon) and this finding, combined with the eosinophilic quality of the cytoplasm, caused these tumors to often be mistaken in the past for smooth muscle tumors. GISTs were also not uncommonly mistaken for neural tumors because they can exhibit prominent nuclear palisading, mimicking a nerve sheath tumor, and can occasionally (approximately 5%) stain positively for S100 protein, a marker common to neural tumors. However, unlike other mesenchymal tumors of the gastrointestinal tract, the vast majority (95%) of GISTs are positive for the tyrosine kinase receptor *c-kit* (KIT) (also known as CD117), typically in a diffuse cytoplasmic,

dotlike, or membranous pattern (31,32). This KIT immunopositivity reflects the effect of activating mutations in KIT (see following section) and has greatly improved the reproducibility of GIST diagnosis.

In the gastrointestinal tract, KIT is also expressed in interstitial cells of Cajal (ICC), gut pace-maker cells exhibiting smooth muscle and neuronal differentiation ultrastructurally and immunohistochemically, which are important for intestinal peristalsis (33). Given the similarities between ICCs and GISTs both ultrastructurally and immunohistochemically, it is believed that most GISTs show differentiation toward ICC.

Genetics and Molecular Pathogenesis

Approximately 85% to 90% of GISTs harbor activating *KIT* mutations (31). The proto-oncogene KIT is a type III receptor tyrosine kinase related to platelet-derived growth factor receptor (PDGFR), and is required for melanogenesis, myelopoiesis,

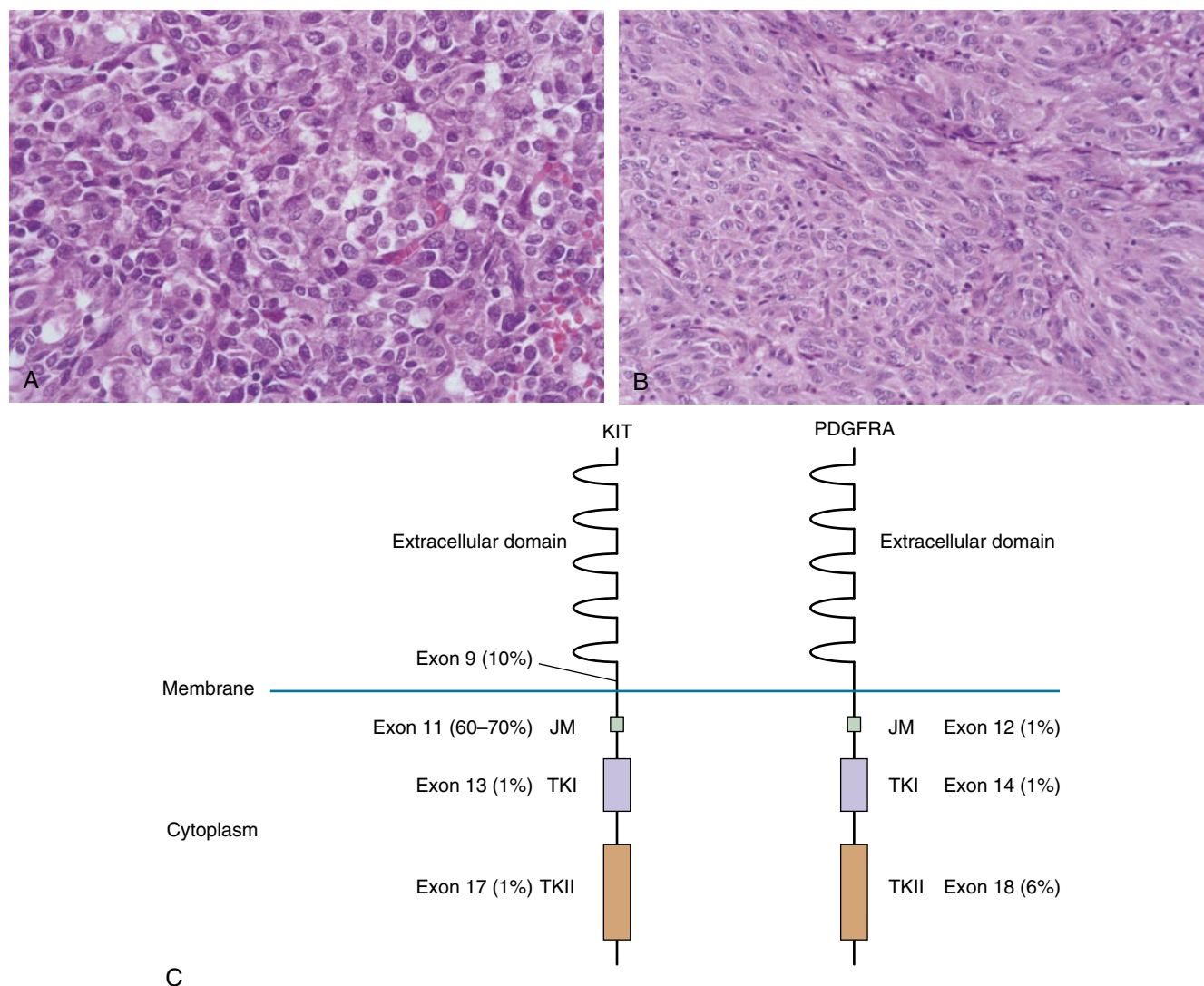


FIGURE 37-2 GASTROINTESTINAL STROMAL TUMOR (GIST). **A:** Epithelioid GIST, showing cells with round nuclei, perinuclear vacuoles, and moderately abundant eosinophilic fibrillary cytoplasm. **B:** Spindle cell GIST, demonstrating the typical monomorphic cells with vesicular chromatin and pale eosinophilic syncytial-appearing cytoplasm, growing in short fascicles. **C:** Diagram of *c-Kit* and platelet-derived growth factor receptor A (PDGFRA) receptors demonstrating localization and frequency of mutations occurring in sporadic GIST. Approximately 5% to 10% of GISTs lack *c-Kit* or PDGFRA mutations. JM, juxtamembrane domain; TKI, tyrosine kinase domain I; TKII, tyrosine kinase domain II.

fertility, and ICC and mast cell development (34,35). It is a transmembrane protein whose ligand is stem cell factor (SCF). Binding of SCF to KIT results in KIT activation via autophosphorylation, leading to downstream phosphorylation of key signal transduction proteins and regulation of a number of cell processes including proliferation, survival, cell adhesion, and differentiation (36). In GISTs, *KIT* mutations identified cluster into four exons, exon 9 (extracellular juxtamembrane domain), exon 11 (intracellular juxtamembrane domain), exon 13 (initial portion of the kinase domain), and exon 17 (kinase activation loop; Figure 37-2C). Data suggest that exon 11 mutations are the most frequent (60%–70% of GISTs) (31). Mutations in exons 13 and 17 are rare.

Platelet-derived growth factor receptor A (*PDGFRA*) is mutated in approximately 5% of GISTs, resulting in constitutive activation and downstream activation of signal transduction molecules similar to those affected by *KIT* activating mutations (37). *KIT* and *PDGFRA* mutations are mutually exclusive and the mutations found in *PDGFRA* map to similar domains on the protein to those found in *KIT* (Figure 37-2C). GISTs with *PDGFRA* mutations tend to exhibit epithelioid rather than spindle cell morphology and tend to be more common in the stomach.

A mouse knock-in model expressing constitutively active *KIT* has demonstrated that this constitutive activation is necessary and sufficient for GIST tumorigenesis (38); thus, mutation in either *KIT* or *PDGFRA* is likely an early step in GIST pathogenesis. However, other recurrent genetic changes have been shown to occur. These include loss of chromosomes 14q, followed by loss of 22, 11p, 9p, or 1p (31). Loss of 9p, on which the tumor suppressor *p16^{Ink4a}* is located, appears to be associated with a worse prognosis. Gains of chromosome 5p, 20q, 17q, or 8q are also associated with a more aggressive clinical behavior; however, correlation of tumor progression with specific genes in those regions has yet to be demonstrated.

Approximately 5% to 10% of GISTs lack *KIT* or *PDGFRA* mutations. Whether these tumors harbor mutations in downstream targets of *KIT* or *PDGFRA* or in a receptor tyrosine kinase similar to *KIT* and *PDGFRA* remains to be seen.

GISTs also occur in the setting of specific genetic syndromes, namely neurofibromatosis type I/von Recklinghausen neurofibromatosis (NF-1), Carney triad, and familial GIST syndrome. In the latter syndrome, patients have germ-line activating mutations in *KIT* or *PDGFRA*, which are inherited in an autosomal dominant fashion (39). These patients also have ICC hyperplasia and those with specific mutations in exon 11 of *KIT* also have abnormal skin pigmentation and mastocytosis. The Carney triad consists of extra-adrenal paragangliomas, pulmonary chondroma, and multifocal epithelioid GISTs of the stomach (40). None of the patients who have been molecularly characterized have germ-line *KIT* or *PDGFRA* mutations. Less than 10% of patients with NF-1 develop GIST but when they do, their tumors tend to be multicentric and are associated with ICC hyperplasia. Interestingly, analysis of *KIT* and *PDGFRA* in NF-1-associated GIST so far suggests that only a minority have *KIT* (9%) or *PDGFRA* (6%) mutations (41,42).

GISTs are not responsive to conventional chemotherapy or radiation and thus, before the identification of *KIT* mutations

in GIST and the development of targeted therapy, the outlook for patients with clinically aggressive GIST was grim. Imatinib mesylate, also known as Gleevec, binds to and inhibits the ATP-binding pocket of both *KIT* and *PDGFRA* and has been approved to treat patients with metastatic and/or unresectable GIST. Mutational status appears to be important in predicting response to Gleevec, in that patients with exon 11 *KIT* mutations have a significantly better response than patients with exon 9 *KIT* mutations or without *KIT* or *PDGFRA* mutations (43). Unfortunately, a significant number of patients eventually develop resistance to Gleevec, typically via secondary *KIT* or *PDGFRA* mutations, or through *KIT* amplification (31). Development of novel tyrosine kinase inhibitors, for example sunitinib (44), to combat this resistance is underway.

KIT has been shown to activate a number of key signal transduction pathways, including the mitogen-activated protein kinase (MAPK), Janus kinase/signal transducers and activators of transcription (JAK/STAT), Src family of tyrosine kinases (SFK), Ras/extracellular regulated kinase (Ras/Erk), and PI3 kinase signaling pathways; however most of these data have been acquired from hematologic systems (36). Of these pathways, preliminary in vitro data demonstrate that the PI3 kinase and MAPK pathways are activated in cultured GIST cell lines (45). In contrast, phosphorylation of the JAK/STAT kinases STAT1 and STAT3 was not dependent on oncogenic *KIT* signaling, and STAT5 phosphorylation was not detected in either primary GIST or GIST cell lines. Another signal transduction molecule, protein kinase C (PKC) θ , a protein important in skeletal muscle signal transduction, neuronal differentiation, and T-cell activation, was also shown to be strongly expressed and constitutively activated in GIST (31), although its role in GIST pathogenesis remains to be elucidated. In hematologic diseases, there is evidence for specific activation of particular pathways depending on the particular *KIT* mutation present. Whether this is true of GIST remains to be determined, and will likely have important treatment implications, particularly for the development of treatment strategies in imatinib-resistant tumors.

Future Directions

The identification of specific chromosomal aberrations in a significant subset of sarcomas has improved both our ability to diagnose and develop novel treatment strategies for these tumors. Just as the elucidation of specific translocations in another category of mesodermally derived neoplasms—leukemias—allowed the development of directed chemotherapeutics (e.g., chronic myelogenous leukemia and Gleevec), so too the discovery of specific recurrent genetic mutations in sarcomas provides opportunities for intelligent design of targeted therapies. This is in contrast to the two thirds of sarcomas that lack recurrent genetic abnormalities, which unfortunately include most pleomorphic/spindle cell sarcomas of older adults. Like most carcinomas, they possess often-complex karyotypes and abnormalities in multiple genetic pathways, making it difficult to pinpoint initiating carcinogenic events. These sarcomas pose a greater challenge to the elucidation of their molecular

pathogenesis and their treatment. Unfortunately, gene expression profiling of these pleomorphic sarcomas in an attempt to achieve reproducible molecular signatures and identify gene targets have failed to yield reproducible results (46). The accrual of additional

and larger expression data sets as well as use of these data in the identification of drug-resistance pathways, prognostic factors, and new therapeutic targets in these aggressive sarcomas is an area of ongoing research.

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Head and Neck Squamous Cell Carcinoma

Epidemiology, Clinical Presentation, and Treatment

Head and neck squamous cell cancers (HNSCCs) arise from the epithelial lining of the upper aerodigestive tract and occur in approximately 40,000 individuals annually in the United States (1). The most common sites are the oral cavity, pharynx, and larynx. HNSCCs account for 85% to 95% of neoplasms of the head and neck. Cigarette smoking and alcohol use are widely considered to be the most important risk factors for head and neck cancer, appear to interact in a synergistic manner with regard to risk (2), lead to a high rate of medical co-morbidity and second primary cancers in these patients (3), and warrant attention as part of any HNSCC risk-reduction strategy. Infection with human papillomavirus (HPV-16 is of special concern) is associated with the development of head and neck cancer, particularly within the oropharynx (4). Cumulative data over 40 years have established a link between Epstein-Barr virus and nasopharyngeal carcinoma (5).

Descriptions of head and neck cancers generally focus on their local-regional character and associated symptoms. Figure 38-1 illustrates the leveling system used to facilitate communication about the location of neck nodes. The location of a lymph node in the neck may direct the clinician to the primary site (e.g., cancers of the oral cavity typically spread to lymph nodes in the submental and submandibular areas [level I]; laryngeal cancer, to the upper part of the neck [levels II and III]). For most primary sites, clinically detectable distant metastatic disease at presentation is uncommon.

The American Joint Committee on Cancer (AJCC) has established stage groupings for all primary sites based on tumor-node-metastasis (TNM) classification (6). T, N, and M categories are combined in an identical fashion for all sites except the nasopharynx. The clinical stage, based on the clinical examination and radiographic information, is more commonly used than pathologic (surgical) staging for treatment planning and result reporting. Most patients with less advanced disease (stage I or II) are cured with single-modality treatment (i.e., surgery or radiation). Patients with locoregionally advanced, stage III or IV disease have a poorer prognosis and require combined modality therapy (i.e., surgery and/or radiation, with or without chemotherapy). Chemotherapy administered by itself is considered in most instances

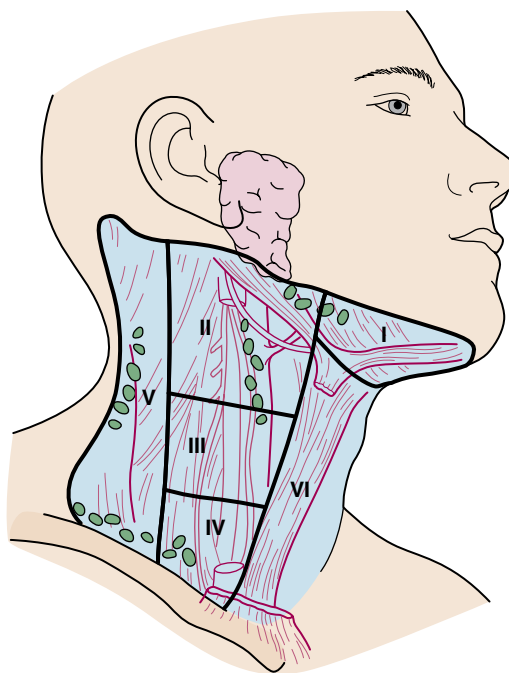


FIGURE 38-1 DIAGRAM OF LEVELS 1 THROUGH 6 FOR CERVICAL LYMPHATIC METASTASES.

to be a palliative treatment, accounting for the grim prognosis associated with the development of distant metastatic disease (stage IVc).

Field Cancerization: Multistep Carcinogenesis Model and Chemoprevention

The field cancerization concept was initially proposed by Slaughter in 1953. The authors observed multiple primary carcinomas in 11% of their surgical specimens, and demonstrated that oral cancer develops in multifocal areas of precancerous change (3).

Subsequent work has confirmed that premalignant genetic lesions may be found throughout the mucosa of the upper aerodigestive

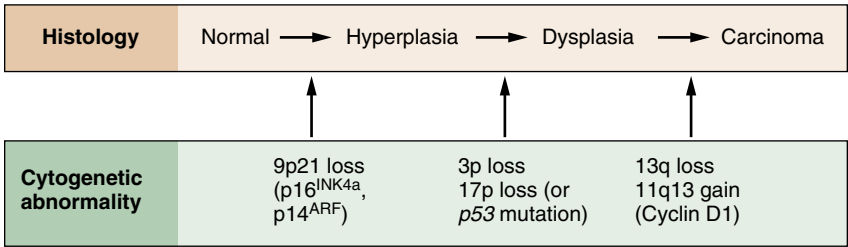


FIGURE 38-2 Hypothetical multistep model for head and neck squamous cell carcinoma (HNSCC) carcinogenesis. This simplified model highlights cytogenetic abnormalities at locations known to encode tumor suppressor genes or oncogenes (shown in parentheses). Only lesions for which the chronology is best established are shown. (From Perez-Ordóñez B, Beauchemin M, Jordan RCK. *Molecular biology of squamous cell carcinoma of the head and neck*. J Clin Pathol 2006;59:445–453, with permission.)

tract in HNSCC patients. Figure 38-2 presents a possible genetic progression model for HNSCC. However, it should be recognized that there likely is variability in the precise sequence of events of carcinogenesis among patients. Some well-characterized cytogenetic chromosomal alterations are presented in Table 38-1 (7,8). Subsequent sections categorize genetic lesions according to functional capabilities they confer on tumor cells, with the caveat that the biologic functions of certain molecules may include more than one category.

The concept of multistep carcinogenesis has attracted interest in chemoprevention strategies to interrupt the process in patients at risk for HNSCC. In mouse models of oral carcinogenesis, retinoids reverse premalignant lesions (9). Clinical responses to isotretinoin in premalignant oral lesions may be associated with restoration of expression of retinoic acid receptor- β mRNA (10), supporting the clinical study of retinoids for chemoprevention. In studies of aerodigestive tract cells, retinoids also have been shown to decrease cyclin D1 expression and cell proliferation (11). However, a large randomized clinical trial of low-dose isotretinoin did not demonstrate efficacy in terms of preventing second primary HNSCCs (12). Other abnormal signaling pathways in premalignant lesions have been described in chemoprevention research. For example, cyclooxygenase-2 expression is increased in the oral mucosa of active smokers, and may be due to an epidermal growth factor receptor (EGFR)–mediated mechanism (13).

Oncogenes

Epidermal Growth Factor Receptor

The EGFR, encoded on the short arm of human chromosome 7, is the prototype of the erbB/HER family of type I receptor tyrosine

kinases. Increased EGFR activity appears to be an early event in HNSCC carcinogenesis. In premalignant oral cavity lesions, higher levels of expression of EGFR and its ligand transforming growth factor α (TGF- α) have been associated with high-grade dysplastic lesions. Overexpression of EGFR is found adjacent to HNSCC tumors in dysplastic tissues. In these nonmalignant tissues, there appears to be an association between dysplastic grade and increased levels of EGFR and TGF- α mRNA and protein (14).

By immunohistochemistry, EGFR expression varies widely among HNSCCs, but approximately 95% of tumors have detectable EGFR. There is no universally accepted definition of EGFR overexpression in HNSCC, and various groups have classified 40% to 65% of HNSCC tumors as EGFR overexpressors. High levels of tumor EGFR expression are associated with worse prognosis in HNSCC (15).

Upon activation, EGFR family receptors trigger diverse intracellular signaling pathways mediating proliferation, cell survival, invasion and motility, and angiogenesis. Regarding cellular proliferation, EGFR activates the Ras-MAP kinase pathway. Phosphorylated EGFR serves as a docking site for Grb2 and other proteins containing Src homology 2 (SH2) domains. Ras, which binds to Grb2 adaptor proteins, phosphorylates Raf kinases, which signal downstream to members of the MAP kinase family. This pathway leads to up-regulation of cyclin D1, permitting cell cycle progression from G₁ to S (16). Another well-characterized pathway downstream of EGFR is the PI3K/Akt pathway, which mediates survival and resistance to apoptosis. EGFR enhances cellular survival by activating phosphatidylinositol-3-kinase (PI3K), which generates the lipid second-messenger phosphatidylinositol 3,4,5-P₃ (PIP₃). Akt kinase is activated by PIP₃, and subsequently activates the anti-apoptotic transcription factor nuclear factor- κ B (NF- κ B). Additionally, Akt inactivates pro-apoptotic proteins such as Bad and caspase 9 (16).

Despite the detailed descriptions of EGFR signaling pathways, the genetic basis for enhanced EGFR activity in HNSCC remains an area of intense investigation with obvious therapeutic implications. Early reports using the polymerase chain reaction found EGFR gene amplification in 15% to 20% of HNSCC patients. Given the interest in EGFR tyrosine kinase domain somatic mutations in non-small cell lung cancer, several groups have analyzed HNSCC tumors for such mutations. But activating EGFR kinase domain exon 19 deletions were found in only three (7%) of 41 HNSCC tumors in one report from Korea (17), and the incidence of EGFR kinase domain mutations in HNSCC patients in Western populations appears to be even lower (18).

Table 38-1 Selected Chromosomal Losses and Gains in Head and Neck Squamous Cell Carcinoma

Event	Incidence	Candidate Genes
Loss		
9p21	70%	CDKN2A encoding p16 ^{INK4a} , p14 ^{ARF}
3p12–24	50%–70%	RASSF1A; others
18q21–23	60%	GALR; others
Gain		
11q13	20%–30%	CCND1 encoding Cyclin D1
3q26.3	40%	PIK3CA encoding PI3K α ; others

Phosphatidylinositol-3-Kinase

PI3K is an intracellular lipid kinase that phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3). An important role of PIP3 is activation of Akt, an anti-apoptotic serine/threonine kinase. *PIK3CA* encodes the p110 α catalytic subunit (PI3K α), and is located on chromosome 3q26.3, which is one of the most common areas of gain in HNSCC (7). The kinase and helical domains of *PIK3CA* are known to harbor oncogenic mutations in other solid tumors, and the incidence of such mutations in HNSCC appears to be approximately 10%, although the number of samples analyzed at this time is small (19).

Cyclin D1

Overexpression of cyclin D1 is the best-characterized abnormality of cell cycle regulation in HNSCC. Cyclin D1 helps mediate phosphorylation of Rb, allowing for cell cycle progression from G₁ to S phase. Cyclin D1 assembles with cyclin-dependent kinases to mediate cell cycle progression, in part by down-regulating p27, which normally functions as a brake on cell cycle progression. Overexpression of cyclin D1 in HNSCC is thought to be due to gene amplification at chromosome 11q13 and has been associated with a worse prognosis (20).

Signal Transducers and Activators of Transcription

Signal transducers and activators of transcription (STATs) are transcription factors that, upon becoming tyrosine-phosphorylated, dimerize and migrate to the nucleus where they recognize specific DNA elements and mediate transcription of genes involved in cell proliferation, differentiation, and apoptosis.

In HNSCC, STAT3 appears to be activated downstream of TGF- α /EGFR as an early event in tumorigenesis. STAT activity may also result from tyrosine phosphorylation by Src kinase, given that direct physical association of c-Src with STATs has been demonstrated in HNSCC 1483 cells (21). Inhibition of STAT3 activity with antisense oligonucleotides or with dominant negative mutants reduced HNSCC cell growth in vitro (22). High tumor levels of phosphorylated STAT3 have been associated with lower patient survival rates (23).

Src Tyrosine Kinase

Src, the first described human oncogene (24), encodes a nonreceptor tyrosine kinase that attaches to the cytoplasmic aspect of the plasma membrane. Activated Src phosphorylates STAT molecules on tyrosine residues, inducing the formation of STAT homodimers and heterodimers. The dimers migrate to the nucleus to activate transcription of genes that mediate cell proliferation. In addition, Src-mediated disruption of the focal adhesion complexes, leading to increased cellular motility, is an early step in the process of cancer cell invasion. Key signaling steps include: Src $\rightarrow$ $\uparrow$ phospho-FAK $\rightarrow$ $\uparrow$ phospho-paxillin (Figure 38-3; 24).

Preclinical studies suggest that Src regulates HNSCC cell proliferation and invasion. Inhibition of Src activity in human HNSCC 1483 cells, with specific Src inhibitors or with a dominant negative mutant *c-Src*, reduced cell proliferation and invasion in comparison with control cells (21,25). In studies with dasatinib, a small-molecule inhibitor of Src family kinases, the IC₅₀ for cell proliferation was less than 100 nM for four of eight HNSCC cell lines (26). Cell migration and invasion were inhibited in all eight HNSCC cell lines, independent of any effects on cell proliferation and survival. Inhibition of Src activity with dasatinib reduced levels of phosphorylated FAK and paxillin (26).

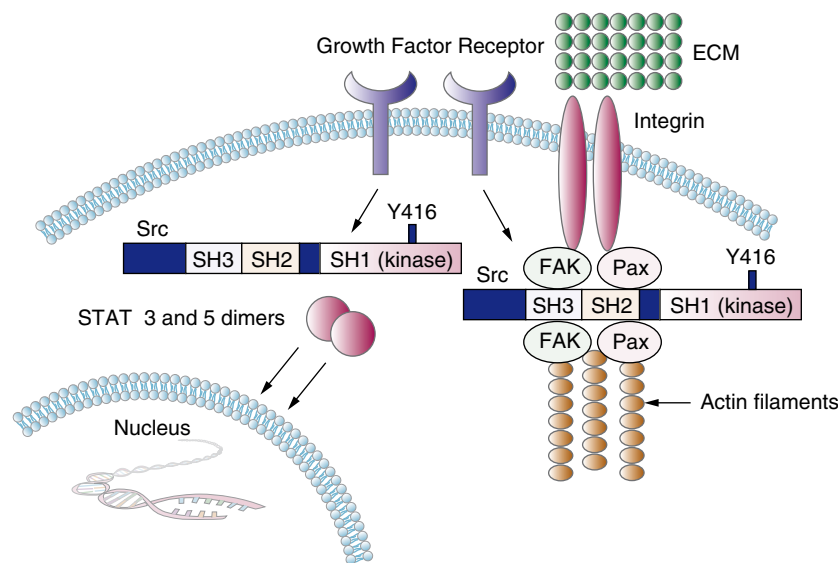


FIGURE 38-3 Schematic illustration of Src kinase signal transduction pathways that have been shown to mediate proliferation and invasion in head and neck squamous cell carcinoma (HNSCC) cell lines. Conserved Src homologous (SH) domains are illustrated within Src.

Synergism between Src and EGFR may contribute to a more aggressive phenotype in multiple human tumors, including HNSCC. Physical association of EGFR and Src has been described in human squamous A431 cells (27). Co-activation of EGFR and Src in the human HNSCC 1483 cell line appears to be modulated by the gastrin-releasing peptide receptor (25).

Tumor Suppressor Genes

p53

The nuclear phosphoprotein *p53* functions as a tumor-suppressor, in part by mediating the cellular DNA damage response. In the canonical mechanism of *p53*-mediated response to DNA damage, *p53* activation results in cell cycle inhibition and prevention of apoptosis, so that the cell may repair DNA damage. The cell will undergo apoptosis if the DNA damage is too extensive for repair. The key signaling molecule downstream of *p53* is the cyclin kinase inhibitor p21, which inhibits cell cycle progression and allows for repair of DNA damage.

An association between conventional risk factors for HNSCC, tobacco and alcohol use, and *p53* somatic mutation was demonstrated in a retrospective study of 129 HNSCC patients. The incidence rate of *p53* mutation was 58% in patients who smoked cigarettes and used alcohol, 33% in patients who smoked but abstained from alcohol, and 17% in patients who neither drank alcohol nor smoked (28). For HNSCC patients without a history of tobacco or alcohol risk factors, loss of *p53* activity may result from infection with human papilloma virus-16 (HPV-16) (4). Inactivation of *p53*, due to somatic mutation in smokers and drinkers or from HPV-16 infection in patients without these risk factors, appears to be an important component of HNSCC tumor biology.

There are a wide variety of *p53* somatic mutations that may be functionally important, and many of these mutations result in increased *p53* signal on immunohistochemical assays. Most somatic mutations occur in exons 5–9, but functionally important mutations may occur at other locations in the gene as well, complicating efforts to comprehensively assess *p53* genetic status in tumors. As such, many HNSCC studies used *p53* immunohistochemical staining as a marker of tumor *p53* status, although it is recognized that the correlation between the immunohistochemical result and *p53* genetic status is imperfect. Results regarding the relationship of *p53* immunohistochemical staining and clinical outcomes have been inconsistent (29, 30), perhaps reflecting methodologic variability or the limited utility of immunohistochemistry as an assay for *p53* functional status.

The incidence of *p53* mutations in HNSCC has been reported to be in the range of 44% to 68% (31,32). As with other solid tumors, most mutations are found in exons 5–8 and approximately 20% of mutations are found elsewhere in the gene. The importance of *p53* in the biology of HNSCC was suggested by surgical series demonstrating that, for patients with histologically negative margins, the presence of *p53* mutations at the surgical margin was associated with an increased risk of tumor recurrence (33).

Several studies have investigated relationships between *p53* mutation status in the primary tumor and clinical outcomes in HNSCC. Analysis for *p53* mutations among 44 patients participating in the Department of Veterans Affairs Laryngeal Cancer Cooperative Study revealed that *p53* mutation was associated with decreased survival (34). In a review of HNSCC patients receiving induction chemotherapy, tumors with *p53* mutation were less likely to respond to chemotherapy (32). For HNSCC patients receiving primary radiotherapy, the results are inconsistent regarding a potential correlation between *p53* mutation and radioresistance.

Given the central role of *p53* in HNSCC biology, gene therapy strategies have been developed to exploit this target. For example, Onyx-015 is a replication competent adenovirus lacking the *E1B* gene, which inactivates cellular *p53*. In a phase 2 study for patients with recurrent HNSCC, intratumoral injection of Onyx-015 led to necrosis in *p53* wild-type tumors (35). In phase 2 and 3 studies of Onyx-015 or the similar virus H101, intratumoral injection of virus improved the efficacy of systemic chemotherapy in HNSCC, independent of tumor *p53* status (36,37).

The *CDKN2A* Locus: p16^{INK4a} and p14^{ARF}

Loss of chromosomal region 9p21 occurs in approximately 70% of HNSCCs (7). This region contains the *CDKN2A* locus, which encodes for p16^{INK4a} and p14^{ARF}. The function of p16 is to provide important indirect support for retinoblastoma (pRB) tumor suppressor protein. Specifically, p16 inhibits the ability of the cyclin D1/cyclin-dependent kinase complex to inactivate pRB via phosphorylation. Whereas active hypophosphorylated pRB blocks the onset of S phase by suppressing the program of the E2F-1 transcription factor, inactive phosphorylated pRB is unable to block the G₁ to S phase transition in the setting of p16 loss. In a study of surgically resected squamous cell carcinomas of the anterior tongue (n = 148), 54% of patients were negative for p16 by immunohistochemistry, and lack of p16 staining was a significant predictor of shorter overall survival by multivariate analysis (38).

P14 is a tumor suppressor that works in coordination with *p53*. The normal function of p14 is to allow for induction of *p53* in response to DNA damage. *p53* activity is constrained by the negative regulator mdm2, but p14 inactivates mdm2. When the ability of p14 to constrain mdm2 is lost, the normal *p53* damage response is also hindered. Retrospective immunohistochemical analysis of surgically resected squamous cell carcinomas of the anterior tongue (n = 140) demonstrated p14 negativity in 20%. In multivariate analysis, p14 negativity was associated with poor overall survival (39).

Phosphatase and Tensin Homologue

Phosphatase and tensin homologue (PTEN), encoded by a gene located at chromosome 10q23, functions as a tumor suppressor by counteracting the activity of PI3K. PTEN has many substrates, but its most important reaction is the dephosphorylation of PIP3 to PIP2. Absence of functional PTEN in cancer cells leads to constitutive activation of downstream components of the PI3K survival pathway, including Akt and mTOR kinases.

Loss of PTEN activity by genetic deletion or somatic mutation occurs in approximately 10% or fewer of HNSCC specimens. Immunohistochemical analysis of primary squamous cell carcinomas of the tongue ($n = 41$) revealed lack of PTEN staining in 29% of the tumors, and PTEN negativity was associated with shorter overall survival in multivariate analysis (40). The authors suggested that other mechanisms of PTEN inactivation may be relevant, possibly including epigenetic events or altered transcriptional or translational processing.

Invasion and Metastasis

Integrins are transmembrane glycoprotein receptors composed of an α subunit and a β subunit, which serve as receptors for extracellular matrix proteins such as collagens, laminin, and fibronectin. Compared with normal mucosa, expression of integrins is altered in squamous cell carcinomas, although expression patterns within a tumor are heterogeneous (41). The integrin that has been most heavily implicated in HNSCC is $\alpha_6\beta_4$, where it appears to activate PI3K to support tumor cell migration (42).

Angiogenesis

The role of angiogenic factors in many solid tumors, including HNSCC, has become an active area of study. Vascular endothelial growth factor A (VEGF-A) is the most potent growth factor for endothelial cells and a strong vascular permeability-enhancing agent. VEGF-A is a ligand for the tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), which are expressed in endothelial cells during development, wound healing, and in tumor vasculature. VEGFR-2 signaling plays a central role in tumor angiogenesis, whereas VEGFR-1 signaling may induce the expression of vascular-bed-specific growth factors and/or modulate the activity of VEGFR-2. The central role of VEGF-A in angiogenesis is demonstrated by the observation that knock-out of a single VEGF-A allele in mice embryos resulted in embryonic lethality between day 11 and day 12 (43).

Increased VEGF-A expression is associated with a poor prognosis in HNSCC. In a meta-analysis of 12 studies that evaluated VEGF-A expression in HNSCC ($n = 1,002$ patients), VEGF-positivity (defined as positive stain in at least 20% of the tumor cells in continuous scales or at least moderate staining in qualitative scales) was associated with a 1.88-fold higher risk of death at 2 years (44).

VEGF-A is one of many circulating mediators of angiogenesis, including VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF), and fibroblast growth factor (FGF). VEGF-C preferentially binds to VEGFR-3, and this ligand-receptor interaction is thought to support lymphangiogenesis. VEGF-C and VEGFR-3 can be detected by immunohistochemistry in most HNSCC specimens. Several groups have suggested that the VEGF-C/VEGFR-3 axis may mediate spread of disease to lymph nodes in the neck. In a retrospective study of 60 HNSCC tumors, VEGF-C mRNA expression correlated positively with lymph node metastasis (45).

Viral Pathogenesis in Head and Neck Cancers: Human Papilloma Virus and Epstein-Barr Virus

HPV (particularly subtype 16), a member of the family of double-stranded DNA papovaviruses, appears to have a role in HNSCC carcinogenesis, particularly among patients who do not have significant history of exposure to tobacco and alcohol. Risk factors for oral HPV infection, such as a high number of sexual partners, appear to increase the risk for HNSCC associated with HPV-16 (4). The HPV-driven mechanism of cellular transformation appears to be distinct from the field cancerization model associated with tobacco and alcohol in HNSCC. Inactivations of p53 and pRB tumor suppressor functions by viral E6 and E7 proteins, respectively, are important steps in squamous cell malignant transformation mediated by HPV (46). This appears to be the preferred mechanism of p53 inactivation in HPV-positive tumors, because somatic p53 mutations are uncommon in HPV-positive tumors (47–52).

In a meta-analysis of 60 studies with 5,046 HNSCC cancer specimens, approximately 26% of specimens were positive for HPV by polymerase chain reaction (53). HPV-positive HNSCC appears to have a better prognosis, compared with HPV-negative HNSCC. In two retrospective studies with over 250 HNSCC patients each, multivariate analyses identified HPV-positivity as an independent predictor of decreased risk of death from cancer (47,54).

Nasopharynx carcinoma (NPC) is an Epstein-Barr virus (EBV)–associated malignancy. NPC is uncommon in the Western world, but is endemic in Southern China, other parts of Southeast Asia, and the Mediterranean basin. EBV is a double-stranded DNA herpesvirus that infects B-lymphocytes and pharyngeal epithelium. Preinvasive lesions of the nasopharynx harbor clonal EBV infections (55). In EBV-infected NPC, the viral gene expression patterns are consistent with latent infection (56).

A prospective study of 99 patients with locoregionally advanced NPC demonstrated that pretreatment plasma EBV DNA levels increase with TNM stage, and higher pretreatment EBV DNA levels are associated with poor prognosis (57).

Future Directions

Key areas of research in HNSCC include the development of chemoprevention regimens for at-risk populations, further elucidation of HNSCC subgroups with distinct tumor biology mechanisms, and the development of treatment regimens with decreased toxicities and improved efficacy. Novel biologic agents targeting oncogenic signaling pathways are anticipated to figure prominently in the development of chemopreventive and cancer treatment regimens, as the fields of molecular prevention and molecular therapeutics will likely have many targets of shared interest.

Clinical treatment paradigms do not reflect the molecular heterogeneity of HNSCC. The identification of HPV-16–associated HNSCC demonstrates that there may be disease subgroups with distinct molecular pathology mechanisms and prognostic

behaviors, with obvious implications for therapeutic targeting and development. The ability to identify HNSCC subgroups would improve the ability to design and interpret clinical studies. Gene expression profiling strategies of the primary tumor are of interest in this regard. For example, a microarray study demonstrated distinct gene expression profiles for HNSCC tumors with nodal involvement versus tumors without nodal involvement (58).

Better systemic therapy is sorely needed. With contemporary surgical techniques, radiation therapy technology, and combined modality approaches, locoregional control rates are improving. As a result, patterns of failure are changing, with an increased incidence of distant metastases that needs to be addressed. Current drug therapy is unsatisfactory for patients with recurrent HNSCC no longer amenable to further surgery or radiation. The median survival for patients in this setting is less than 1 year.

The successful development of cetuximab in HNSCC motivates further rationally designed clinical studies of therapeutic agents that target specific molecular pathways. Inhibition of other erbB family members in HNSCC merits further investigation

because the family members dimerize to form potent signaling complexes. The PI3K/Akt/mTOR pathway appears to drive the malignant phenotype of a significant subset of HNSCCs. Although specific inhibitors of PI3K have not entered advanced-stage clinical development, mTOR inhibitors (CCI-779 and everolimus) have shown efficacy in other tumors types and await further study in HNSCC.

With the increasing understanding of tumor–stromal cell interactions in HNSCC, strategies to inhibit tumor cell invasion and angiogenesis will be explored in the clinic. For example, small-molecule inhibitors of the Src tyrosine kinase have entered clinical development in various malignancies, and it is hoped that such drugs may help control the locally invasive phenotype of HNSCC. Anti-angiogenesis therapies are now standard options for selected solid-tumor types, and the potential role of these agents in HNSCC is being investigated. If any of these classes of agents demonstrate clinical activity in HNSCC, additional clinical research would determine if such agents allow for the modification of current combined modality regimens to reduce toxicity and improve efficacy.

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Primary Brain Tumors

Malignant primary tumors of the central nervous system (CNS) occur in about 20,500 individuals and account for an estimated 13,000 deaths in the United States annually, a mortality rate of 6 per 100,000. Based on the most recent report of the Central Brain Tumor Registry of the United States, benign tumors of the CNS occur in numbers comparable to malignant brain tumors with a much lower mortality rate (1). Overall, CNS cancer is estimated to represent about 1% of newly occurring malignant tumors. In children and young adults, brain tumors are a major health problem second only to leukemia as a cause of cancer-related deaths, and they are the third leading cause of cancer-related deaths between ages 15 and 34. The age-adjusted incidence appears to be about the same worldwide.

Brain tumors are a diverse group of neoplasms. The particular combination of somatic genetic alterations found in different brain tumors that are histologically distinguishable can vary considerably among individual examples of a particular tumor type. Recognition of a number of hereditary syndromes in which brain tumors play a prominent role provides compelling evidence for the importance of several cell regulatory pathways important in brain tumor pathogenesis (Table 39-1). Primary CNS tumors have been classified by surgeons and pathologists on the basis of their location and histologic appearance, providing important information that guides treatment with conventional antineoplastic modalities including surgery, irradiation, and chemotherapy as well as contributing to prognostication. Benign CNS tumors consist primarily of meningioma. Glial tumors account for 50% to 60% of all primary brain tumors, and with the exception of some pilocytic astrocytoma, most are malignant. Astrocytomas account for the great majority of the glial tumors, whereas the second most common is oligodendroglioma. Exposure to ionizing radiation is the only well-documented environmental risk factor for the development of glioma.

Hereditary Syndromes and Central Nervous System Oncogenesis

In addition to rare families in which there is strong evidence for a hereditary basis for the development of familial meningioma or glioma, a number of well-studied cancer predisposition syndromes

include among their associated stigmata the development of CNS tumors (Table 39-1). These are important clinical observations and have provided insights into the pathogenesis of several different types of primary brain tumors. Typically these syndromes are associated not only with an increased incidence of brain tumors, but also with the occurrence of tumors at an earlier age than those arising spontaneously and with the finding of multiple tumors in a single individual. To date, all syndromes that include brain tumors are inherited as autosomal dominant disorders and arise as the result of the germ-line mutation of one allele of a tumor-suppressor gene whose other copy is typically inactivated in the tumors that arise. The most commonly occurring brain tumor predisposition syndrome is neurofibromatosis type 1 (NF1; 2). NF1 is an autosomal dominant disease effecting approximately 1/3000 live births approximately 15% of whom have radiographic evidence of optic glioma early in childhood, although most cases do not become symptomatic. Although peripheral nervous system tumors are common in NF1 patients, these patients are also at increased risk of developing pilocytic astrocytomas and, more rarely, malignant astrocytomas. The protein product of the *NF1* gene, located on the long arm of chromosome 17, is neurofibromin 1 (NF1), which functions to antagonize the proliferative function of p21^{ras}. Neurofibromatosis type 2 (NF2) is distinct from NF1, affecting approximately one in 50,000 individuals (3). The *NF2* gene has been localized on the long arm of chromosome 22 encoding a protein known as neurofibromin 2 or merlin (NF2). NF2 patients frequently present before the third decade of life with deafness caused by bilateral schwannomas of the eighth cranial nerve. Less commonly NF2 patients develop neuronal schwannoma including tumors of the spinal and cranial nerves, meningioma, low-grade astrocytoma, and ependymoma. The Li-Fraumeni syndrome is observed in patients with germ-line *TP53* mutations (4). Although affected family members are predisposed to a number of different tumor types, brain tumors are among the more common and include astrocytomas, medulloblastomas, and choroid plexus tumors. The *TP53* protein product, p53, plays an important role in the DNA damage checkpoint of cells and in regulating apoptosis. Its function is commonly inactivated by mutation in a wide variety of human tumors and is thought to play an important role in sporadically occurring astrocytic tumors.

Table 39-1 Hereditary Syndromes Associated with Brain Tumors

Syndrome	Gene	Chromosomal Location	Associated Central Nervous System Tumors
Neurofibromatosis type 1	<i>NF1</i>	17q	Optic glioma
Neurofibromatosis type 2	<i>NF2</i>	22q12	Acoustic schwannomas, meningioma, ependymoma, glial tumors
Retinoblastoma	<i>RB</i>	13q14	Retinoblastoma, pinealoblastoma
Li-Fraumeni syndrome	<i>P53</i>	17p13	Glioma, medulloblastoma
von Hippel-Lindau syndrome	<i>VHL</i>	3p	Retinal angioma, cerebellar hemangioblastoma
Tuberous sclerosis	<i>TSC1</i> <i>TSC2</i>	9q 16p13	Giant cell subependymal astrocytoma, astrocytoma, ependymoma
Turcot syndrome	<i>APC</i> , <i>hMSH1</i> , <i>hMSH2</i> , <i>hPMS2</i>	5q21	Astrocytomas, medulloblastoma
Gorlin syndrome	<i>PTCH1</i>	9q22.1-q31	Medulloblastoma, astrocytoma

Molecular Biology of the Most Common Primary Central Nervous System Tumors

Brain tumors are thought to arise as the result of genetic and epigenetic alterations that activate oncogenes and inactivate tumor suppressor genes (Table 39-1). The precise cell of origin in which the most common brain tumors arise is unknown, but emerging evidence suggests these tumors arise as a result of the malignant transformation of neural stem cells or very early precursor cells or that more differentiated cells take on key characteristics of neural stem cells as a result of their malignant transformation (5). Early progenitors have many of the characteristics that seem integral for tumor development—proliferative potential, migratory capacity, and multipotentiality—but definitive studies have not ruled out the possibility that tumors arise in association with dedifferentiation of more mature, more highly differentiated cells. Interestingly, either of these origins could provide an explanation for the most commonly occurring primary tumors of the CNS including diffusely infiltrating astrocytoma, oligodendroglioma, medulloblastoma, and meningiomas, which are described in the following sections. Recognition of a small fraction of glioma cells with features of neural progenitor cells and a tumor-initiating function indicates the presence of glioma tumor stem cells.

Astrocytic Tumors

Diffusely infiltrating astrocytomas are so named because they display morphologic and some biochemical evidence of glial differentiation. These are the most common tumors of adults and children, occur throughout the CNS, and exhibit a wide range of histopathologic appearances and clinical behaviors. These tumors are organized by the World Health Organization (WHO) according to tumor grade (6). Histologically, malignancy is manifested as hypercellularity, cellular atypia, endothelial proliferation, necrosis, and invasion of normal adjacent tissue. WHO grade I tumors are variants of astrocytoma that are generally benign, and WHO

grade IV, also known as glioblastoma multiforme (GBM), is the most aggressively malignant (Figure 39-1). Prognosis is closely associated with pathologic grade (6). WHO grades II and III exhibit intermediate grades of malignancy, but the evidence of increased mitotic activity in grade III tumors is likely to be a key contributor to poor prognosis. All pathologic grades of glioma can exhibit

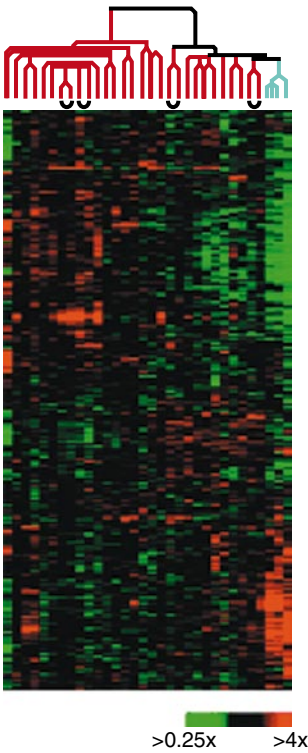


FIGURE 39-1 Microarray evaluation of gene expression and dendrogram demonstrating distinctive groups of glioblastomas (GBMs; red) and normal brain (blue). Individual tissues are aligned in columns and individual genes in rows. Colored pixels capture the level of gene expression where shades of red and green represent induction and repression relative to the median for each gene. Black pixels reflect no change from the median, and gray pixels represent missing data. (From Liang Y, Diehn M, Watson N, et al. Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme. *Proc Natl Acad Sci U S A* 2005;102:5814–5819, with permission. Copyright 2005 National Academy of Sciences, U.S.A.)

invasiveness, and this characteristic compromises the possibility of treating these tumors with surgery alone. The known propensity of some astrocytic tumors that present as low-grade tumors to progress over time to higher grade tumors has provided insight into the pathogenesis of these tumors suggesting that they are closely related and that progression is associated with the acquisition of sequential genetic alterations (Figure 39-2 and Table 39-2). High-grade astrocytic tumors, GBMs, that arise in this manner are called secondary GBMs. The finding of selected genetic changes (e.g., *TP53* mutation) in lower grade tumors that are also present in higher grade tumors along with additional mutations typically found only in higher grade tumors (e.g., epidermal growth factor receptor [*EGFR*] amplification) suggests that specific genetic alterations are associated with particular pathologic features characteristic of the corresponding grade. Genetic changes currently thought to be important in this regard are shown in Figure 39-2 and Table 39-2, an adaptation of the pathogenesis model first proposed for colon cancer (7). Although amplification of *EGFR* is found in approximately 50% of GBMs, this change is rarely found in WHO grade III and never in grade I or II tumors. Also, small deletions and rearrangements of the *EGFR* gene are commonly found in GBM (8). Up to 50% of tumors in which amplification of *EGFR* is detectable also have a rearrangement of *EGFR* in which exons 2 to 7 are deleted (9). This results in an in-frame deletion that encodes a constitutively active *EGFR* (*ERBB2*) against which both antibodies and small-molecule therapeutics are being developed. The possible prognostic importance of *EGFR* amplification in GBM has been the most extensively examined marker in brain tumors, and several research groups have found that *EGFR* amplification in tumor cells was associated with a poor outcome.

Mutation of the *TP53* gene is likely to be an early event associated with the change of normal cells to low-grade neoplasia.

Table 39-2 Tumor Suppressor Genes and Proto-Oncogenes Implicated in Brain Tumor Development

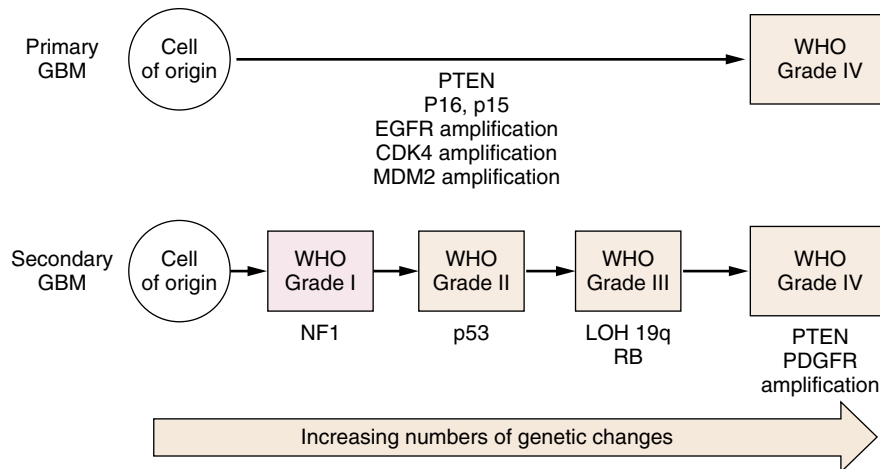
Tumor-Suppressor Gene		Proto-oncogene	
Gene or Locus	Chromosomal Location	Gene or Locus	Chromosomal Location
<i>VHL</i>	11q13, 3p26-p25	<i>EGFR</i>	7p12
<i>TSC1, TSC2</i>	9q34; 16p13.3	<i>PDGFR-A</i>	5q31-q32
<i>PTCH1</i>	9q22.3		
<i>REST</i>	Chr. 4	H-ras, N-ras	11p15; 1p13
<i>CDKN2A, CDKN2B</i>	9p21	c-myc, N-myc	8q24; 2p23-p24
<i>P53</i>	17p13.1	<i>MDM2</i>	12q14
<i>NF2</i>	22q12		
<i>NF1</i>	17q11.2		
<i>RB1</i>	13q14		
<i>APC</i>	9q31		
<i>PTEN</i>	10q23		

Commonly mutated residues are codons 248 and 273 (10). *p53* has an important role in stabilizing the genome, and the genetic instability resulting from its loss may contribute to the accumulation of multiple mutations in a single cell that are required for the development of highly malignant tumors. Inactivation of *TP53* by mutation and epigenetic mechanisms may occur in up to 75% of astrocytomas. *MDM2*, encoding MDM2, a protein that inhibits the ability of *p53* to promote transcription by targeting the protein for degradation, is amplified in approximately 10% of gliomas and these invariably have a wild-type *TP53* gene. Loss of heterozygosity (LOH) for Ch9p21 at the site of the *CDKN2A* and *CDKN2B* loci results in homozygous deletion of these adjacent genes in approximately 60% of GBM. This deletion results in the loss of the p15 (INK4B), p16 (INK4A), and p14 (ARF) tumor-suppressor proteins. *CDKN2A* and *CDKN2B* encode cyclin-dependent kinase inhibitors, p16 (INK4A) and p15 (INK4B), respectively, which bind to the cyclin-dependent protein kinases CDK4 and CDK6 inhibiting the catalytic activity of the cyclin D-cdk complex. P14 (ARF) is expressed from a distinct transcript as the result of alternative splicing of the *CDKN2A* gene, and functions to keep MDM2 in the nucleolus so that it cannot degrade *p53*. Loss of p14 (ARF) results in degradation of *p53*. Other cytogenetic changes including +7p/q, +19q, and -11p/q are widely recognized in high-grade brain tumors, but the best understood is clearly the deletion of chromosome 10 where *PTEN* is located (11). The *PTEN* protein product, PTEN, is a lipid phosphatase that antagonizes the function of the phosphatidylinositol 3-kinase (PI3K) family of lipid messengers and consequently inhibits downstream signaling through AKT1, a serine/threonine kinase that is a key regulator of critical cell functions including cell proliferation and survival.

Several different receptor tyrosine kinases (RTKs) and their cognate ligands have been implicated in the malignant behavior of astrocytic tumors, and especially GBM. These include PDGF and PDGFR, EGF and EGFR, TGF- α , and EGFR as well as IGFR and others. Although members of the PDGF pathway tend to be overexpressed in all grades of astrocytic tumors, amplification and mutation are rare. Activation of RTKs in glioma leads to autophosphorylation of these receptors, which in turn leads to the docking of a series of proteins on phosphorylated sites of the receptors and downstream activation of various effectors. Activation of RAS by the docking protein SOS1 leads to initiation of the RAF/MEK/MAPK (ERK) pathway that leads to the transcriptional activation of genes important for proliferation. Docking of GAB1 to another phosphorylated site on autophosphorylated RTKs leads to activation of PI3K, which in turn initiates a cascade of phosphorylation leading to PDK1 phosphorylation of AKT1. Activated AKT1 in turn phosphorylates both a number of pro-apoptotic proteins, inactivating them, and a series of transcription factors that are thereby activated and lead to transcription of other genes important for cellular proliferation. About 80% of GBMs exhibit activation of AKT1, primarily as a result of RTK activation or deletional inactivation of *PTEN*.

Not all GBMs exhibit the same constellation of genetic alterations and, interestingly, a second clinical presentation of GBM seems to be associated with a distinctive genetic profile (Figure 39-2). Approximately 10% of GBMs present without evidence of

FIGURE 39-2 Glioblastoma arises in association with the accumulation of multiple genetic alterations. Primary glioblastoma manifests *de novo* without the presentation of any precursor lesion. Secondary glioblastoma results from the accumulation of additional oncogenic mutations by lower grade astrocytic tumors resulting in increased malignancy.



a precursor lesion (de novo, primary GBM). Although high levels of *EGFR* expression are frequently found in primary GBM, these are rarely detectable in secondary GBM (approximately 10%). In primary GBM, amplification of *MDM2* is a more common strategy for inactivation of *TP53* than it is in secondary GBM. The *CDKN2A* and *PTEN* loci are also frequently inactivated by mutation in these tumors.

Gene-expression profiling to enhance pathologic classification of glial tumors and identify new targets for therapy and new biomarkers of prognostic significance is an important ongoing line of investigation (Figure 39-1; 12–14). Although most studies have examined only small numbers of tumors, it has been possible to recognize clinically and molecularly distinguishable subsets of glioblastoma and to authenticate the efficacy of FABP7 as a prognostic marker for these patients (14).

Oligodendroglioma

Oligodendrogliomas account for about 20% of primary intracranial tumors and are the second most common glial neoplasm (1). Most frequently these tumors occur in the cerebral hemispheres typically within the white matter. They are characterized by various histologic criterion of malignancy, which determines their grade. These tumors can have a highly variable spectrum of differentiation, and their cellular origins are not understood. There is frequent confusion over the proper diagnostic classification for tumors that do not display prototypical characteristics of cells arising in the oligodendroglial lineage or contain multiple populations of cells, one of which will correspond to the oligodendroglial lineage and the other to an astrocytic lineage. Histopathologically, tumors of the oligodendroglial lineage exhibit round, homogeneous, compact cells with a dense central nucleus and clear cytoplasm, sometimes referred to as a “fried egg” appearance. There is rarely identifiable evidence of mitosis. When tumors are highly anaplastic with obvious necrosis and mitotic figures, they are indistinguishable from GBM and are designated as such. Different histologic grades of oligodendroglioma have recently been associated with specific patterns of gene expression (15), which raises the possibility not only of novel biomarkers and therapeutic targets for this tumor but

also suggests that the molecular basis for tumor progression will become a tractable problem.

The cytogenetic and molecular biologic profile of oligodendrogliomas is less well characterized but apparently quite distinct from that of astrocytoma (16,17). Cytogenetic studies of oligodendroglioma may reveal loss of chromosomes 9p and 22 or gain of chromosome 7, but the most characteristic cytogenetic findings in oligodendroglioma are loss of chromosomes 1p36 and 19q13.3. Recently it has been proposed that this loss of large amounts of DNA is mediated by an unbalanced $t(1;19)(q10;p10)$ (18,19). The presence of these alterations is associated with an enhanced responsiveness to cytotoxic therapies and an improved prognosis (20). LOH for chromosome 9p, observed in some oligodendrogliomas, corresponds to deletions at the *CDKN2A* locus and these have been associated with a poor prognosis for patients with this tumor type. Studies looking for mutations that are frequently found in astrocytic tumors have not detected commonly occurring changes in the *TP53* locus. Similarly Ch10 losses and *EGFR* amplification have only been rarely detected in these tumors. These distinguishing molecular genetic differences are consistent with more recent genetic expression analyses that reveal clear differences between astrocytoma and oligodendroglioma.

Meningioma

Meningiomas account for approximately 20% of primary intracranial malignancies (1) and are derived from mesoderm, probably from cells giving rise to the arachnoid granulations. These tumors are usually benign and attached to the dura mater. They infrequently invade the brain parenchyma. Meningiomas may be found incidentally on a computed tomography (CT) or magnetic resonance imaging (MRI) scan or may present with a focal seizure, a slowly progressive neurologic deficit, or symptoms of raised intracranial pressure. Total surgical resection of benign meningioma is curative, and when tumor persists, external beam radiotherapy or stereotaxic radiosurgery reduces the recurrence rate to less than 10%. No effective chemotherapy for the treatment of benign meningiomas is known and there is no curative therapy for anaplastic, highly invasive meningiomas that cannot be treated locally.

Meningiomas are an important manifestation of NF2, and in approximately half of sporadic meningioma, portions of Ch22q, which contains the gene encoding NF2 at Ch22q12, is lost. These findings provide strong support for a role of NF2 as a tumor-suppressor gene whose inactivation contributes to the development of meningiomas. NF2 functions as part of a molecular complex linking the cellular plasma membrane to the cytoskeleton, where it has been implicated in the regulation of cellular proliferation. The frequency of NF2 mutation does vary somewhat in different histologic types of meningiomas (21,22). NF2 mutations occur in only about 25% of meningothelial meningioma, whereas more than 75% of fibroblastic and transitional meningioma have been found to have evidence of NF2 inactivation. Other karyotypic abnormalities are also seen in grade II (atypical meningioma), and grade III (anaplastic meningioma). In anaplastic meningioma, the most frequent cytogenetic abnormalities are deletion of Ch1p, partial or complete loss of Ch10q, and loss of Ch14. Unstable chromosome alterations including rings, dicentric, and telomeric associations also have been observed. Meningiomas express a variety of growth factor RTKs and targeted therapeutics such as imatinib are being evaluated for the treatment of anaplastic, invasive meningioma.

Medulloblastoma

Medulloblastoma accounts for about 80% of childhood primitive neuroectodermal tumors (PNETs) of the CNS and accounts for approximately 15% to 20% of all pediatric brain tumors (1). Only gliomas are more common in children. In contrast to other PNET, these tumors are found in the cerebellum where they are thought to arise from cells of the fetal external granular layer (23). Although histologic differentiation of medulloblastoma from atypical teratoid/rhabdoid tumors of the CNS can be difficult, rhabdoid tumors are now recognized as invariably containing either germline or somatic mutations of the *INI1* gene. In contrast, medulloblastoma is readily distinguishable from childhood glioma in that it appears as a highly cellular, small, round cell tumor with frequent mitoses and pseudorosettes. Also, medulloblastoma responds to therapy much more readily than glioma and is very sensitive to radiation and chemotherapy as evidenced by the importance of therapeutic response as a prognostic factor in this disease (24). The 5-year disease-free survival approximates 50% to 70% (25).

As noted in Table 39-1, the occurrence of medulloblastoma in three inherited cancer syndromes: Turcot syndrome, Li-Fraumeni syndrome, and Gorlin syndrome has called attention to the possibility that the genes responsible for these syndromes, *APC*, *TP53*, and *PTCH1*, respectively, or the pathways in which they are active might play a role in sporadic medulloblastoma. Although evidence for *APC* inactivation in sporadic medulloblastoma has not been identified, *TP53* mutations and mutations of *PTCH1* and genes in the *PTCH1*-mediated sonic hedgehog (SHH) pathway are found in sporadically occurring medulloblastoma (26). Deletions of Ch6q and Ch16q have been noted also in medulloblastoma tumor tissues and infrequent amplification of *MYC* and *MYCN* has been detected. *NTRK3* neurotrophin receptor expression in medulloblastoma has been reported to be

associated with a favorable outcome (27), a finding consistent with emerging evidence that medulloblastoma tumor tissue expresses a number of genes associated with the neuronal lineage and that expression profiling of these genes may provide a particularly effective approach to devising clinically useful prognostic biomarkers (28,29).

Molecular Pathophysiology of Primary Central Nervous System Tumors

Brain tumors present therapeutic challenges that reflect their occurrence within the closed space of the skull and within a tissue that is particularly sensitive to disruption of its normal function. As a result, simple growth as a space-occupying lesion is particularly problematic in the development of brain tumors as is the invasion of normal tissue, which can occur early in the pathogenesis of some tumor types such as primary GBM and much later in others. The proliferation of brain tumors is thought to be the result of the deregulation of oncogenes and tumor suppressor genes as described previously, and many of the growth stimulatory pathways that contribute to tumorigenesis in the CNS have been extensively examined. In addition to the oncogenetic alterations described for individual tumors, important regulatory roles in the proliferation of brain tumors include those mediated by EGF and PDGF, which are widely expressed in the normal brain as well.

Gliomas and medulloblastomas are typically highly invasive, and this characteristic is thought to play an important role in their recurrence after treatment that tends to occur locally at their original site of presentation. The mechanisms that underlie glial tumor cell invasion are an active area of research (30–32). Clinicians observed long ago that such malignant tumors have preferred routes of invasion along white matter tracts, and degradation of the extracellular matrix by proteinases produced by tumor cells is now recognized as an important early step in the invasion of normal tissue, although little is known of the manner in which tumor cells recognize white matter tracts and migrate along them. Glial tumor cells express urokinase-type plasminogen receptor (UPAR) on their surface (33), which binds to urokinase plasminogen activators (UPAs) that are also highly expressed in the most malignant gliomas (34). UPAR presents activated UPA to the extracellular matrix (ECM) where plasminogen is cleaved to active plasmin, which degrades ECM constituents including fibronectin, laminin, and proteoglycans that are especially prominent in the ECM of blood vessels. Interestingly, some brain tumors produce inhibitors of various plasminogen activators, thereby inhibiting ECM degradation. The extracellular milieu of the CNS also contains high concentrations of glycosaminoglycan, hyaluron, and chondroitin sulfate, which are detected in only low amounts in other tissues. How brain tumor cells move through a matrix of such molecules is not known.

Development of a tumor vasculature is a central aspect of tumorigenesis and tumor progression (35). Although low-grade brain tumors tend to have a vasculature similar to that observed in normal brain, high-grade tumors are heavily vascularized and disrupt the established blood-brain barrier and lack such as a

barrier in the new tumor vessels that are formed (36). Glial tumor vessel formation is regulated by the endothelial cell RTK for VEGF, including FLT-1 (VEGFR1) and KDR (VEGFR2; 37). VEGF is a critical mediator of tumor angiogenesis in several different types of brain tumors, and its expression is hypoxia-inducible, a finding consistent with cellular hypoxia being a key stimulant for angiogenesis during glioma formation. VEGF is expressed at very high levels in high-grade gliomas (37). FLT-1 is not expressed in normal brain tissue but is expressed in a tumor-stage-dependent manner in the vasculature of astrocytoma, and KDR is expressed only in high-grade glioma (38). VEGF is also known to enhance vascular permeability of normal brain vasculature and may be responsible for the peritumoral edema found frequently in malignant gliomas. Blood vessel formation during tumorigenesis and tumor progression requires both proliferation and recruitment of smooth muscle pericytes, which is regulated by TGFB1, the PDGFRB, and TEK (TIE-2). The TEK receptor and its agonist, ANGPT1, are constitutively expressed in gliomas, suggesting an important role in the development of tumor vasculature for this pathway as well. Currently, nonspecific inhibitors of angiogenesis, such as suramin, and specific inhibitors, such as TNP-40 and Avastin, are being evaluated for the treatment of GBM in clinical trials.

Therapeutic Resistance of Primary Central Nervous System Tumors

Primary brain tumors are widely regarded as being particularly resistant to the most commonly used antineoplastic strategies. Although surgery plays a major role in removing some brain tumors and debulking others, the challenges of working within the skull in a tissue so exquisitely sensitive to anatomic disruption mean that tumors that either arise in or invade into regions of the brain that are required for vital functions cannot be effectively removed. Also, aggressive brain tumors, especially high-grade astrocytomas, tend to invade as innumerable projections from the surface of the tumor and as rare cells that move to very distant regions of the brain, making complete removal impossible. Similarly, the use of radiation therapy is challenged by the compactness of normal tissues in the vicinity of the tumor and by the invasion of tumor into normal tissue. These characteristics of brain tumors mean that delivering effective doses to the tumor without damaging critical normal tissues is particularly difficult. This is especially important in the developing brain, and compromises our ability to use the highest doses of radiation therapy in children.

Both radiation therapy and chemotherapy are compromised because many glial-derived tumors seem to be particularly resistant to apoptosis following DNA damage (39). Although the precise contribution of apoptosis to curative therapy in solid tumors is not yet known, it is clear that tumors that are highly resistant to apoptosis do not respond well to cytotoxic therapies. Also, the blood-brain barrier presents a unique challenge for the administration of chemotherapy. Although the barrier is largely destroyed at sites of primary tumor growth, migrating, invasive tumor cells are thought to remain behind the blood-brain barrier until they begin to establish millimeter size masses, and this finding may account for the apparent resistance to therapy of some tumor types. The use of combination therapy consisting of procarbazine, lomustine (CCNU), and vincristine (PCV) has been found to be quite active in a subgroup of patients with oligodendroglioma, which is characterized by the loss of Ch1p and Ch19q (40). Also, the combination of temozolamide with radiation has resulted in a slight increase in median survival of patients with GBM, a disease in which surgery and radiation alone result in approximately 1-year survival duration (41). These results indicate that cytotoxic approaches can contribute to the treatment of brain tumors, and hopefully will provide insights into how multimodality therapy might be more effective in the future.

Future Directions/Perspective

Molecular analysis of brain tumors opens important opportunities to identify specific genetic changes that are of pathophysiologic importance. Clearly in the case of high-grade gliomas, different molecular subgroups can be identified. Initially, it seems likely that molecular characterization will focus on prognostic classification. Targeted therapy is being pursued as a goal of conventional drug development seeking small-molecule therapeutics and novel strategies such as antisense oligonucleotides, small inhibitory RNAs, and ribozymes. Other novel strategies that are being pursued and have already reached clinical trial include the use of conditional oncolytic viruses (42), viruses carrying prodrug-activating genes (43), recombinant virus that synthesize antitumor agents (44), and the inoculation of autologous neural stem cells as delivery vehicles for tumor-toxic molecules to target tumor cells that have spread far from the primary tumor site (45). There is also considerable interest in the development of antibody and immune-mediated therapies for glioma. Vaccines will be of particular interest if it is possible to treat residual disease following conventional therapy (46,47). Molecular classifications of the future should seek to classify brain tumors on the basis of specific alterations that might be significant therapeutic targets.

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Epithelial Skin Cancer¹

Skin Cancer Research has Helped Define the Biology of Cancer Pathogenesis

Epithelial skin cancer (primarily basal cell and squamous cell carcinoma) is the most frequent cancer among white populations, with incidence rates matching all other cancers combined in these groups. Although not frequently lethal, epithelial skin cancers are the cause of considerable morbidity, cosmetic defects, and extreme medical expense. Exposure to mutagenic ultraviolet (UV) light is the major skin carcinogenetic stimulus, but sun avoidance has often been superseded by life-style choices, and changing atmospheric conditions favor increased intensity of exposure. Thus, considerable attention has been directed and progress achieved for understanding the molecular pathogenesis of epithelial cutaneous cancer to develop effective preventive and therapeutic approaches that are easily adopted by the affected individuals. Beyond the practical importance of skin cancer research for human health lays the value that such research has provided for understanding cancer pathogenesis in general. No other target tissue has provided such panoply of insights in this regard. Centuries ago, astute clinicians and basic scientists first observed that chemical and physical exposures could cause skin cancer in humans and animals. Modern analysis of skin cancer induction demonstrated that cancers evolve from multiple, clonal, precancerous stages (initiation, promotion, progression, conversion), and that genetic (e.g., mutation), epigenetic (e.g., gene expression or protein modification) and microenvironmental changes (e.g., inflammation, wounding) contribute to tumor formation. Skin studies first revealed that chemical carcinogens bind covalently to DNA causing specific mutations in oncogenes and suppressor genes, and that DNA repair protects against cancer risk. Additionally, cutaneous cancer research has indicated that viruses have potential to cause human tumors, immunosuppressed patients are susceptible to cancer, genetic background can modify cancer rates, and diet and cigarette smoke are cancer risk factors. Thus, epithelial skin cancer research has been a major contributor to defining cancer biology and biochemistry in general.

Genetic studies on familial basal cell cancer have further revealed that the hedgehog signaling pathway contributes to the pathogenesis of a variety of epithelial cancers of internal organs (1).

The Molecular Origin of Skin Tumors is Revealed by Hereditary Syndromes

Cutaneous cancers are the manifestation of germ-line mutations in a number of hereditary syndromes (Table 40-1). Identifying the mutant genes has revealed pathways relevant to the pathogenesis of cancers in skin and internal organs as well as revealing targets for somatic mutations in spontaneous versions of the same tumor type. The most broadly relevant are the DNA repair genes that comprise the complementation groups of skin cancer prone xeroderma pigmentosum families (2). Germ-line mutations in individual genes at distinct chromosomal loci define proteins that collectively insure proper global and transcription coupled nucleotide excision repair. Among these are proteins that recognize and bind to sites of DNA damage (XPA, XPC, XPE), helicases (XPB, XPD), and endonuclease components (XPG, XPF), defects in any of which give a skin cancer prone phenotype. From this lead, it is now recognized that constitutive polymorphisms among sun-exposed individuals may alter repair efficiency and lead to enhanced susceptibility to skin cancer and other cancers in the general population (2). The discovery of germ-line mutations in the *PTCH1* gene among patients with the basal cell nevus syndrome first revealed the involvement of the Sonic hedgehog pathway in human cancer (1). Subsequent studies confirmed that most sporadic basal cell cancers had somatic mutations in *PTCH1* or its downstream effector *SMO* (reviewed in [3]). The mapping of the dysplastic nevus syndrome to the *INK4a* locus and identification of mutations in the *p16^{INK4a}* gene revealed an important pathway in the etiology of heredity-prone and sporadic melanoma (4). As importantly, this discovery pointed to defects in structure or expression of *p16^{INK4a}* and other components of the cyclin-CDK signaling pathway in melanoma and nonmelanoma skin cancer (4). Specific

¹ We have attempted to adhere to standard nomenclature guidelines (http://www.nature.com/ng/web_specials/nomen/nomen_guidelines.html) through most of the text. Human genes and proteins are indicated in upper case, with only the gene name italicized (e.g., *PTCH1* and PTCH1). For mouse homologues, only the first letter in each is upper case (*Ptch1* and *Ptch1*).

Table 40-1 Gene Targets for Mutations in Hereditary and Sporadic Cutaneous Cancers

Gene	Function	Locus	Tumor Type	Syndrome	Spontaneous
<i>p53</i>	DNA repair, apoptosis, cell cycle regulation	17 p13.1	BCC, SCC	Li Fraumeni (but no increase in skin cancers)	Yes
<i>XPA, XPB, XPC, XPD, XPE, XPF, XPG</i>	DNA repair	9p34.1, 2q21 3p25.1, 19q13.3, 11p11.12 16p13.3, 13q32	BCC, SCC, melanoma	Xeroderma pigmentosum	Possible
<i>PTCH1</i>	Sonic hedgehog receptor	9q22.3	BCC, trichoepithelioma	Nevoid basal cell carcinoma	Yes
<i>SMO</i>	Sonic hedgehog effector	7q31-32	BCC	Not identified	Yes
<i>p16^{INK4a}</i>	Cyclin inhibitor	9p21	Melanoma, SCC, trichoepithelioma	Dysplastic nevus	Yes
<i>CTNNB</i> (β -catenin)	Cell-cell adhesion, transcription factor	3p22-p21.3	Pilomatricoma	Not identified	Yes
<i>CYLD1</i>	NF- κ B inhibitor	16q12-13	Cylindroma Trichoepithelioma	Multiple cylindroma	Yes
<i>PTEN</i>	Phosphatase	10q23.3	Trichilemmoma	Cowden	Unknown
<i>MSH2</i>	Mismatch repair	2p22-p21	Sebaceous gland carcinoma	Muir-Torre	Unknown
<i>MLH1</i>		3p21.3			
<i>Folliculin</i>	Unknown	17p11.2	Fibrofolliculoma	Birt-Hogg-Dubé	Unknown
?	?	9p21	Trichoepithelioma	Multiple trichoepithelioma	Unknown
?	?	Xq24-q27	BCC	Bazex	Unknown
?	?	9q31	Keratoacanthoma	Ferguson-Smith	Unknown

?: BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

mutations in Cowden syndrome (*PTEN*), Muir-Torre syndrome (*MSH2*, *MLH1*), pilomatricoma (*CTNNB* [β -catenin]), Birt-Hogg-Dubé syndrome (*FLCN*), cylindromatosis (*CYLD1*) and trichoepithelioma (*PTCH1*, *p16^{INK4a}*) that cause adnexal tumors have revealed much about the pathways important in the development of cutaneous adnexal tissues. Remarkably, these syndromes have also provided important information about associated internal tumors regulated by similar pathways. For example, the hedgehog pathway is implicated in medulloblastoma, pancreatic, prostate, and oral cancers, among others. Folliculin defects are strongly linked to renal cancer, *PTEN* to breast cancer, and *MSH2* to colon cancer.

Cutaneous Cancers Arise from Multipotential Stem Cells

The multiplicity of tumor phenotypes that arise from skin (e.g., Table 40-1 and [5]) suggest that multiple target cells exist within the complex compartmentalization of the integument. Alternatively, tumor lineage and malignant potential are determined by a specific genetic lesion or tumor microenvironment acting on a multipotential target

cell (6). Divergent phenotypes are also abundant within basal cell carcinomas (7) and squamous cell carcinomas (8). Genotyping of both lesions indicate a monoclonal origin favoring a single stem-like cell as the precursor to these major cancers. Both human and murine skins contain a population of clonogenic, multipotential stem cells in the bulge region of the hair follicle. In mice, targeting oncogenic Ras to the hair follicle gives rise to squamous tumors with a high risk for malignant conversion while intraepidermal targeting produces only benign tumors (9). Advances in stem cell isolation have provided gene expression profiles that characterize follicle bulge cells (10). Prominent among these gene expression signatures is members of the Wnt gene family, p63, and specific integrins all capable of regulating lineage specificity. Experimental data in mice support a gene-expression-driven lineage determination. In these studies, modulating the activity of the AP-1 family of transcription factors in benign skin tumors causes reciprocal transdifferentiation between squamous and sebaceous tumor lineages. The lineage switch occurs in a subpopulation of multipotential tumor cells driven by AP-1 regulated expression of Wnt (squamous) or hedgehog (sebaceous) proteins (11). These results suggest a common multipotential precursor cell gives rise to diverse tumor lineages regulated by epigenetic changes in the tumor microenvironment.

Basal Cell Carcinoma

Basal cell carcinoma (BCC) is a common, slow-growing, locally invasive tumor that typically presents as a pink or pearly papule with superficial telangiectasia and occasional ulceration. BCC precursor lesions have not been identified, there is no evidence of neoplastic progression, phenotypic diversity even within the same tumor is common and metastases are exceedingly rare (7). Chromosomal losses involving 9q mark both sporadic and inherited BCCs leading to the discovery of mutations in *PTCH1* (1) a homologue of the *Drosophila ptc* gene involved in embryonic development. Interestingly, a closely related *PTCH2* gene is not associated with BCC development although it may function in skin homeostasis in mice (12), and can modulate tumorigenesis in *Ptch1* mutant mice (13).

Ptch1 is a 12-pass transmembrane receptor for Sonic hedgehog (Shh), a secreted ligand involved in proliferation and patterning of multiple tissues and organs during embryogenesis. Shh initiates signaling by binding to *Ptch* and alleviating the *Ptch* mediated repression of *Smo* on responding cells. *Smo* has homologies to G protein-coupled receptors, and activated *Smo* is phosphorylated by casein kinase 1 and protein kinase A. Active *Smo* influences the expression of a family of Gli transcription factors to alter the expression of Shh target genes (1). The complexity of the pathway is enhanced by the presence of additional vertebrate homologues of *Drosophila* proteins, fused (a serine-threonine kinase), suppressor of fused (*SuFu*), that binds to Gli proteins, *Cos 2* and *Iguana*, with *SuFu*, *Cos2*, and *Iguana* serving as inhibitors of Gli signaling (14). Activation of the Shh pathway up-regulates Gli1 and *Ptch1*, and these serve as markers for physiologic and pathologic Shh signaling. In human BCCs, inactivating mutation or deletion of *PTCH1* results in constitutive signaling independent of SHH. In addition to loss-of-function *PTCH1* mutations, gain-of-function (oncogenic) *SMO* mutations have been found in some BCCs where *PTCH1* appears to be normal (15). *PTCH1* or *SMO* mutations are implicated in about 80% of BCCs while hedgehog signaling is active in all BCCs. Thus, additional mechanisms must exist for uncontrolled activation of this pathway, and a few mutations in *SuFu* have been reported in BCCs (16). Although mutations in p53 have been found in up to 50% of BCCs, most lesions fail to exhibit the genomic instability associated with other cancers where p53 function is compromised (17), and the presence or absence of p53 mutations does not alter the histologic phenotype (18).

The consequences of Gli activation appear to be the driving force for tumor development in the setting of constitutive Hedgehog signaling. The mammalian Gli family is composed of three isoforms, all regulated by hedgehog signaling. Gli proteins are zinc finger proteins that bind to a 9bp canonical binding site in the promoters of target genes (1). The regulation of Gli activity by hedgehog signaling is complex, and Gli2 and Gli3 proteins have both transcriptional repressor and activating domains, while Gli1 is strictly a transcriptional activator. Much of the regulation of Gli proteins is post-translational (19). Gli3 processing yields largely a transcriptional repressor while processed (phosphorylated) Gli2 is ubiquitinated and degraded. Shh signaling suppresses processing

and degradation of Gli2 and stabilizes its transcriptional activation function. Genetic ablation studies in mice have displayed the consequences of Gli activity *in vivo*. *Gli1* null mice are without a phenotype, whereas ablation of *Gli2* causes numerous developmental defects consistent with deficient hedgehog signaling, including severely growth-impaired hair follicles (20) suggesting that Gli2 is the downstream effector in this pathway. Disruption of *Gli3* produces a phenotype consistent with hedgehog activation, validating its action as a repressor of the pathway.

The experimental support linking the hedgehog pathway to human BCC comes from a variety of genetically altered mouse models. Targeting SHH or an activated SMO mutant to the epidermis and hair follicles up-regulates Shh target genes and produces basal cell-like proliferations in newborn mouse skin. Overexpression of SHH in human keratinocytes followed by grafting onto SCID mice produced BCC-like changes as well. Mouse models in which *Ptch* gene function has been disrupted develop microscopic hair follicle-derived proliferations, with the appearance of a variety of macroscopic skin tumors, including BCCs, following exposure to ionizing or UV radiation. Mice with skin targeted overexpression of human GLI1 or mouse Gli2 develop multiple BCC or other tumors arising in hair follicles demonstrating the multipotentiality of cells to which these genes are targeted (1,15). Taken together, these findings strongly support the concept that deregulated Shh signaling is central for BCC development. When Gli2 is targeted to mouse epidermis and hair follicles conditionally, BCCs develop from overexpression and regress when expression is discontinued. Upon re-expression of Gli2 in this model, tumors reemerge from a small residual population of precursor cells (21).

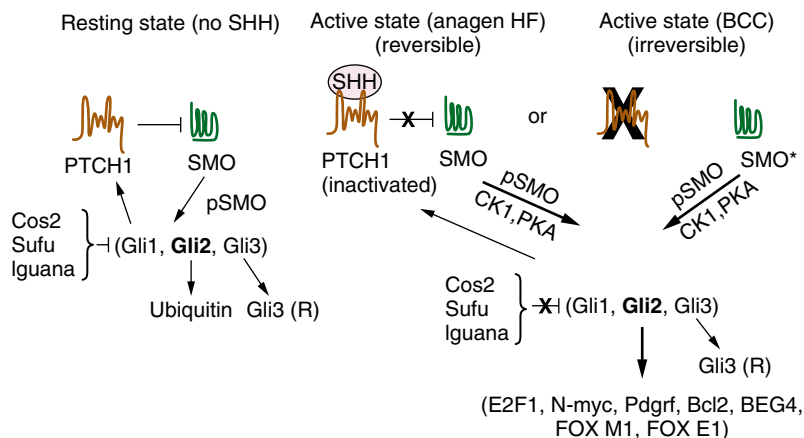
What remains unclear in the story of BCC is the identification of the hedgehog target genes downstream from Gli that are essential for BCC formation. A number of candidates have been reported including several cyclins, *E2f1*, *N-Myc*, *Pdgfr*, *Bcl2*, *BEG4*, *FOXM1*, and *FOXO1*. However, definitive data linking these genes to the unique pathology and diverse phenotypic expression of this common cancer are lacking. Nevertheless, cyclopamine, a natural product inhibitor of SMO, causes BCC regression in animal models, and an analogue is in clinical trials. Thus, the elucidation of the hedgehog pathway as a mediator of BCC development just a few years ago has provided a new approach to treat the most prevalent human cancer. Moreover, since the Hedgehog pathway is aberrantly activated in malignancies arising in several internal organs (1), the fundamental studies addressing basal cell carcinoma may yield knowledge applicable to a broad range of human neoplasms (Figure 40-1).

Cutaneous Squamous Cell Carcinoma

Cutaneous squamous cell carcinoma (SCC) frequently presents as a firm, pink papule or nodule, with a conspicuous hyperkeratotic surface (8). Although they represent only about 20% of non-melanoma skin cancers, SCCs are invasive and occasionally metastasize (1%–2%). SCC is more frequent with higher cumulative sunlight exposure and as cancers associated with specific occupational exposures (coke oven and petroleum oil workers). There is

FIGURE 40-1 Proposed model depicting hedgehog signaling in skin physiology and BCC pathogenesis. The major site of action for sonic hedgehog (SHH) is the hair follicle (HF). In resting hair follicles, SHH is not expressed, and PTCH1 dampens the activity of SMO. Under these conditions, Gli transcription factors are unstable and subject to degradation and inhibition by binding to Cos2, SuFu, and Iguana. Gli3 processing yields a transcriptional repressor (Gli3R). When SHH expression increases during HF growth (anagen), PTCH1 is inactivated, SMO is phosphorylated (pSMO) by casein kinase 1 (CK1) and protein kinase A (PKA), and active SMO up-regulates Gli proteins by post-translational mechanisms. One target of Gli transcription is PTCH1, which serves as a negative feedback on this reversible pathway. In BCC initiation, PTCH1 is genetically deleted (or SMO is mutationally activated to SMO*), and the pathway is irreversibly activated. Gli2 appears to be the major driving factor to up-regulate a number of effectors responsible for the neoplastic phenotype.

THE HEDGEHOG PATHWAY IN CUTANEOUS PHYSIOLOGY AND TUMOR DEVELOPMENT



roughly a 25-fold increase in SCC incidence in immunosuppressed organ transplant recipients, and these tumors are more aggressive, occur in multiple locations within one patient and are associated with increased morbidity and mortality (22). Conversely, a drug that activates local innate and adaptive immune responses through TLR-7 (imiquimod (1-(2-methylpropyl)-1H-imidazo [4,5-C] quinolin-4-amine) is highly effective in treating BCC, SCC, and its precursor lesion, actinic keratosis (23). Cutaneous SCC is usually preceded by a benign hyperproliferative-hyperkeratotic actinic keratosis (AK; Figure 40-2A). These are sunlight-induced clonal lesions that frequently harbor *p53* mutations, particularly at codon 278 or other codons of the DNA binding domain of *p53* that contain dipyrimidine sites. AKs often exhibit chromosomal changes particularly loss of heterozygosity (LOH) at 3p, 13q, 17p, 17q, 9p, and 9q (22). Such changes are less frequent in SCC clouding the direct relation of AK to SCC. However, the frequency of evolution of AK to SCC is very low (0.1%–10%) suggesting there may be a high-risk AK group with relevant genetic changes not yet documented. Activating mutations in the *K-RAS* or *Ha-RAS* gene are detected in approximately 10% of AK and SCC, and the *RAS* pathway is activated by nonmutational mechanisms in a much larger fraction of SCC (22). Inactivating mutations or epigenetic silencing of *p16^{INK4a}* and activation of telomerase are other pathways associated with SCC development (22). Constitutive activation of the EGF receptor (EGFR) by amplification or increased expression of ligands with the formation of an autocrine loop is a frequent finding in SCC (24). Gene expression arrays have revealed several other genes whose expression is characteristic of SCC, but experimental validation of a causal relation remains to be determined.

Constitutive activation of NF- κ B signaling is common in SCC (25,26), and this is associated with up-regulation of specific NF- κ B target genes associated with altered proliferation, invasion, angiogenesis and inflammation. NF- κ B hyperactivation is a known inducer of cylindromatous skin tumors since homozygous deletions of the *CYLD* gene, encoding a deubiquitinase that negatively regulates the NF- κ B pathway through targets such as TRAF2 and BCL-3, (27) has been identified as the basis for

familial cylindromatosis (Table 40-1). Homozygous deletion of *CYLD* in mice increases susceptibility to squamous skin tumors (27). However, studies with genetically modified human keratinocytes grafted to nude mice suggest that inhibition of NF- κ B together with activation of the *RAS* oncogene is sufficient to convert normal keratinocytes into SCC (28), and a similar result has been obtained in the skin of transgenic mice (29). This controversy is unsettled at this time.

To prove causal relations among the various associations made by studying human SCC, model systems using human and mouse keratinocytes in culture and animal models in vivo have been developed. The classical model to induce SCC on mouse skin involves a limited application of a mutagenic agent such as a chemical carcinogen or UV light that “initiates” the cancer process in a subset of cells followed by repeated applications of a nonmutagenic agent such as a phorbol ester that provides a microenvironment favorable for the clonal outgrowth of the initiated population. Tumor development proceeds through predictable stages with the early emergence of benign squamous papillomas, the murine equivalent of AK, some of which progress to invasive SCC, mimicking human SCC with a low metastatic rate (Figure 40-2B). In some cases SCC will evolve into a more aggressive spindle cell cancer. This model has illuminated the genetic and biochemical changes that are permissive for SCC development under controlled experimental conditions (9,22,30). Heterozygous activating *Ras* gene mutations are sufficient to initiate the target cells and produce squamous papillomas, and this is coupled to constitutive activation of the EGFR through overexpression of EGFR ligands (Figure 40-2B). Papillomas form spontaneously in transgenic mice overexpressing *ErbB2* and *TGF- α* in epidermis, and papilloma formation is reduced in mice where EGFR or SOS are ablated (31,32). Furthermore tumor formation is completely inhibited in mice lacking the *Stat3* gene, a downstream target of the EGFR (33). Inactivation of *PKC- δ* is also essential for papilloma development in mouse skin. *PKC- δ* is inactivated by c-Src-mediated tyrosine phosphorylation in initiated mouse keratinocytes whereas in human skin tumors the expression of *PKC- δ* is greatly reduced (34). Transgenic mice overexpressing *PKC- δ* in the skin do not

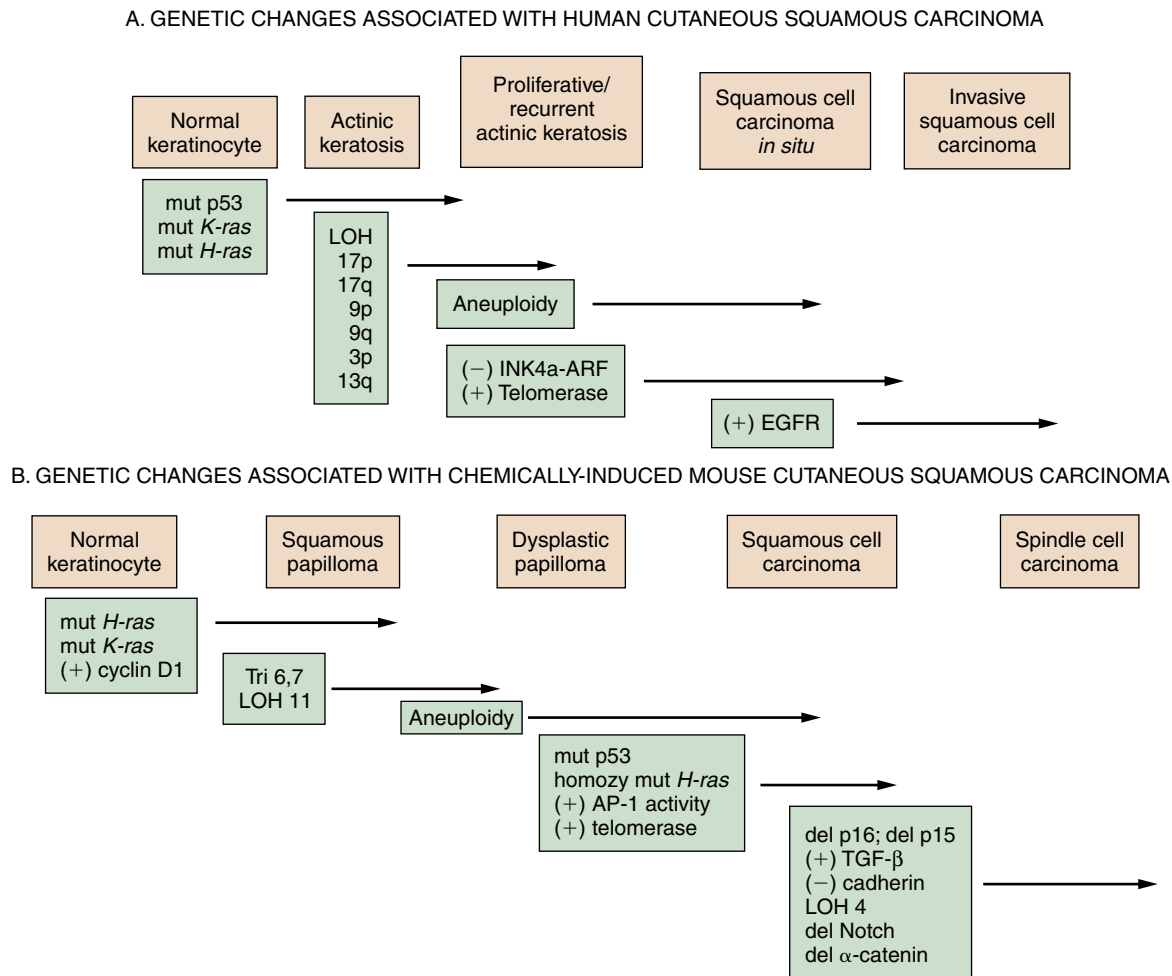


FIGURE 40-2 Genetic changes associated with (A) human cutaneous squamous carcinoma and (B) experimental mouse skin squamous carcinoma. The multistage evolution of invasive squamous cell cancer is depicted schematically with frequently associated genetic changes detailed. Although many common pathways exist in the two species, understanding places *p53* mutations early in human SCC development and UV light-induced mouse skin SCC (not shown), but *p53* mutations occur late in chemically induced mouse skin SCC associated with malignant conversion. Increased activity of telomerase (deletion of inhibitor) or EGFR tyrosine kinase (gene amplification) may also result from epigenetic changes as in the case of up-regulation of EGFR ligand expression in mouse SCC development. (From Dlugosz A, Merlino G, Yuspa SH. *Progress in cutaneous cancer research. J Invest Dermatol Symp Proc* 2002;7:17, with permission.)

form skin tumors (35). TGF- β plays a dual role in experimental SCC development, suppressing premalignant progression to SCC while enhancing phenotypic progression from SCC to a spindle cell phenotype (36). Inactivation of *p53* enhances malignant conversion of papillomas to SCC in chemical carcinogenesis but occurs early in UV light-induced skin carcinogenesis in both mice and humans (9). Members of the AP-1 transcription factor family also play a dual role in experimental skin tumor development, where c-Jun is essential for papilloma and c-Fos is essential for SCC development. Inhibition of AP-1 activity after the formation of benign papillomas prevents malignant conversion (11).

The development of techniques to genetically alter mice greatly expanded the array of genes and pathways that influence SCC development on the skin (Table 40-2). Pathways now implicated in SCC development and progression to spindle cell tumors from experimental studies are cyclin D1, ornithine decarboxylase, *p16^{ink4A}*, *p15^{ink4A}*, *p63*, E-cadherin, and TGF- β 1, and these are altered in human SCC. In several model systems, genetic alterations

have resulted in the spontaneous development of SCC in the absence of a precursor lesion. For example, mice ablated for Notch or α -catenin genes rapidly form SCC even in the absence of carcinogenic exposure (25,37).

Although much cutaneous cancer research has focused on cell-autonomous alterations in keratinocytes that contribute to cancer development, it is clear that alterations in the cutaneous environment are also critical (38). Early studies showed that human SCC and chemically induced SCC and their precursor lesions exhibit altered distribution and expression of the $\alpha_6\beta_4$ integrin complex, a major component of the hemidesmosome (39). In transgenic mouse models, suprabasal overexpression of $\alpha_6\beta_4$ integrin enhanced malignant conversion, whereas overexpression of $\alpha_3\beta_1$ suppressed conversion (40). Similarly, the predisposition to SCC of patients with dystrophic epidermolysis bullosa, a blistering skin disorder is due to specific mutations in the anchoring molecule collagen VII, which blocks the interaction of collagen VII with laminin 5. Introduction of these mutant collagen VII genes into noninvasive malignant

Table 40-2 Genetically Modified Mouse Models for Cutaneous Squamous Cancer

Modification	Enhancers	Comments
Tg.AC (ζ globin- <i>v-ras^{Ha}</i>)	Promoters, drugs	Enhancers up-regulate transgene
Tg.AC	UVB	P53 mutations are absent
K1-ras, K10-ras	Promoters	Predominantly papillomas
Δ K5-ras	None	Papillomas, KA, SCC
K6-ras	Promoters	SCC
K1-TGF α , K14-TGF α , MT-TGF α	Promoters	Predominantly papillomas that regress
Inv-c-MycER	None	Papilloma
K1-v-fos	Promoters	Papilloma
K5-E2F1	P53 deficiency	Papilloma, SCC, BCC
<i>K5-Igf1</i>	Promoters	Papilloma, SCC
<i>K5-ErbB2</i>	Promoters	Papilloma, SCC
K5-SOS-F	None	Tumors inhibited by EGFR deficiency
K14-HPV16	FVB/N mouse strain	Tumors inhibited by difluoromethylornithine
K6-ODC	DMBA	SCC, K-ras mutations
XP mutant models (A, C, D)	Initiation/promotion/UVR	Enhanced sensitivity
<i>Egfr</i> null mutant	<i>v-ras^{Ha}</i>	Reduced tumor size
<i>p53</i> null mutant	DMBA/TPA	Enhanced malignant conversion
<i>p21^{waf1}</i> null mutant	DMBA/TPA	Enhanced papilloma formation
<i>c-fos</i> null mutant	cross with Tg.AC	Papilloma but no SCC
K14-PKC ϵ	DMBA/TPA	Enhanced SCC, metastases
K14-PKC δ	DMBA/TPA	Reduced papilloma development
K5-src	None or DMBA/TPA	Enhanced spontaneous or induced SCC
K5-I κ B mutant	None	Spontaneous SCC/NF- κ B inhibition
Notch null	None	Spontaneous SCC/nuclear β -catenin
α -catenin null	None	Spontaneous SCC/NF- κ B activation
Cyld null	None or DMBA/TPA	Enhanced papilloma/NF- κ B activation

BCC, basal cell carcinoma; DMBA; SCC, squamous cell carcinoma; TPA.

human keratinocytes caused rapid invasion in a graft model that depended on the interaction with the $\alpha_6\beta_4$ integrin (41).

Genetically altered mice have also shown the importance of the immune system in suppressing cutaneous SCC formation. Deletions of key immunoregulatory genes such as interferon- γ (IFN- γ) or perforin enhance chemically induced skin tumor formation. Similarly, mice with a deletion of the T-cell receptor (TCR) δ locus, and thereby lacking the skin resident $\gamma\delta$ T cells, have increased frequency of chemically induced papillomas and malignant conversion, suggesting that this resident T-cell population is important in antitumor immunosurveillance (42). Humoral factors that mediate inflammation such as prostaglandins and TNF- α enhance experimental cutaneous carcinogenesis. Likewise, chronic inflammatory skin conditions such as discoid lupus

erythematosus, dystrophic epidermolysis bullosa, and chronic wounds are associated with increased susceptibility to human skin cancer (43). In these cases, components of the adaptive immune system may also have a tumor promoting effect. For example, mice with a TCR- β deletion (lacking all $\alpha\beta$ T cells) have a significantly reduced carcinoma yield. Thus $\gamma\delta$ and $\alpha\beta$ T cells have nonredundant and potentially opposing roles in tumor development (44).

Perspective

Advances in understanding the molecular basis of cutaneous cancer have reinforced the paradigm that particular genetic and epigenetic changes and the pathways they regulate contribute to skin

cancer formation in a stage specific manner. What benefit may come from these current insights? This is most clear in the case of BCC where delineation of the molecular mechanism of pathogenesis is so precisely defined (perhaps better than any other human cancer) that curative therapeutic targets are identified, and precise animal models have been developed for testing new therapeutics. Cyclopamine and derivatives with better therapeutic index are in clinical trials designed to block SMO with remarkable results, and tazarotene, a retinoid used successfully to treat BCC lesions, is an inhibitor of GLI function. Although BCC is generally not life threatening, the high frequency of these lesions on exposed skin favors a medical rather than the traditional surgical approach, an advance that has been achieved by translation of basic research. Advances in tools for large-scale expression and genomic analysis have been applied to SCC lesions and their precursor AK. Animal models and human tissue analyses have suggested that premalignant precursor lesions vary in risk for progression, and it is anticipated that molecular profiling will reveal markers to identify high-risk lesions for closer clinical scrutiny. Similarly, profiling of SCC will undoubtedly reveal signature markers associated with lesions at risk for metastatic spread. Two molecular therapeutic targets derived from basic research on SCC pathogenesis show promise for medical therapy of SCC. Inhibitors of the EGFR, in clinical use for several internal malignancies, show promise in animal models for the prevention of UV-induced mouse skin SCC. Ingenol-3-angelate (PEP 005), now in clinical trials, targets protein kinase C to induce an innate immune response that destroys the AK, BCC, and potentially SCC lesions. Stimulation of innate immunity to destroy skin tumors is another paradigm for cancer therapy first introduced into the clinic with the drug imiquimod (Aldara), which targets toll-like receptor 7. Other

targets identified from experimental studies that offer therapeutic potential are telomerase, TGF- β , and p53, since drugs targeting these molecules are in clinical trials for treating a number of epithelial cancers.

A developing concept anticipated from molecular analyses is that common gene or protein expression profiles would reveal similar pathogenic mechanisms for skin SCC and squamous tumors of the lung, head and neck, and other sites that pose a threat to life. Similarly, data already exist indicating that the hedgehog pathway is involved in internal malignancies such as pancreatic cancer and others. If such mechanisms are shared, then new drugs could be tested on skin tumors for therapeutic efficacy. The high frequency of skin cancers, the superficial location, and the capacity for topical testing suggest that the skin provides an excellent surrogate site for evaluating drug development for a variety of internal tumor sites. The skin is also a site that often predicts the presence of internal tumors with such lesions as acanthosis nigricans, dermatomyositis, paraneoplastic pemphigus, and other dermatoses. Little is known of the pathogenesis of these premonitory lesions, but undoubtedly such knowledge would reveal important aspects of the host response to cancer. Thus, progress in skin cancer research will continue to provide important translational opportunities not just for these very prevalent lesions but for the advancement of cancer treatment in general.

Acknowledgments

Due to the breadth of this review, we apologize for the unavoidable exclusion of references to work done by many outstanding investigators working in these areas.

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Endocrine Cancer

Most hereditary tumor predisposition syndromes are autosomal dominant, have high penetrance, and are caused by germ-line loss-of-function mutations of a single allele. Multiple endocrine neoplasia type 2 (MEN2) is exceptional in being caused by a germ-line-activating mutation of the *RET* oncogene, a receptor-tyrosine kinase, which plays a pivotal role in thyroid malignancies, particularly medullary thyroid cancer (MTC). The cause of six autosomal dominant endocrine neoplasia syndromes, including multiple endocrine neoplasia type 1 (MEN1), Carney complex (CNC), von Hippel-Lindau disease (VHL), neurofibromatosis (NF1), hyperparathyroidism–jaw tumor syndrome (HPT-JT), and hereditary paraganglioma syndrome (PGL) are germ-line mutations in tumor-suppressor genes. Mutations in tumor-suppressor pathways also contribute to the etiology of pituitary and gastroenteropancreatic tumors. A review of the molecular pathogenesis of the most common endocrine neoplasia is discussed in this chapter, including a differentiation of germ-line versus somatic mutations.

Thyroid Tumors

Thyroid tumors are the most prevalent malignancies of the endocrine system. Follicular-cell–derived tumors comprise benign adenomas and well-differentiated (papillary or follicular), poorly differentiated (e.g., insular), and undifferentiated (anaplastic) carcinomas. Somatic chromosomal rearrangements involving the *RET* gene represent the most frequent genetic alteration in papillary thyroid cancer (PTC) with variations in frequency, ranging from 5% to 70% in different geographic areas. These rearrangements lead to the fusion of the *RET* tyrosine kinase domain with the 5'-terminal regions of heterologous genes, generating chimeric oncogenes designated as *RET/PTC*. To date, 12 rearranged forms of *RET* referred to as *RET/PTC* 1–9, *PCM1-RET*, *ELKS-RET*, and *RFP-RET* have been isolated from sporadic and radiation-associated PTCs. In each case, the intracellular domain of *RET* is fused to different activating genes, namely *H4* (for *RET/PTC1*), *R1a* (*RET/PTC2*), *RFG/ELE1/ARA70* (*RET/PTC3* and *RET/PTC4*), *RFG5* (*RET/PTC5*), *hTIF* (*RET/PTC6*), *RFG7/TF1γ* (*RET/PTC7*), *kinectin* (*RET/PTC8*), *RFG9* (*RET/PTC9*), *PCM1* (*PCM1-RET*), *ELKS* (*ELKS-RET*), and

RFP (*RFP-RET*), resulting in ligand-independent dimerization and constitutive activation of these chimeric proteins (2). *RET/PTC* rearrangements activate the transforming potential of *RET* by multiple mechanisms. First, by substituting its transcriptional promoter with those of the fusion partners, they allow the expression of *RET* in the epithelial follicular thyroid cells, where it is normally transcriptionally silent. Secondly, the rearrangements generate constitutively active chimeric oncoproteins, which are distributed in the cytosolic compartment of the cell. Finally, activation of the *RET* kinase is mediated by fusion to domains that are capable of dimerization. Ionizing radiation can induce *RET/PTC* rearrangements, and thyroid cancer is the most common solid neoplasm associated with radiation exposure (3). In children exposed to radiation, intrachromosomal rearrangements involving *RET* and the adjacent *H4* (*RET/PTC1*) or *RFG/ELE1* (*RET/PTC3*) have been detected in 57% to 87% of tumors removed 5 to 8 years after exposure and in 49% to 65% of tumors diagnosed 7 to 11 years after the Chernobyl accident. *RET/PTC3* rearrangement is strongly associated with PTC of short radiation latency and connected with the solid-follicular variant. Transgenic mice expressing *RET/PTC3* in the thyroid display an aggressive tumor phenotype, including lymph node metastases. The high oncogenic potential of *RET/PTC3* correlates with its prevalence in the aggressive tall-cell variant (TCV) of PTCs.

Activation of *BRAF* serine/threonine kinase by chromosomal rearrangement has been reported in several papillary thyroid carcinomas. The fusion gene derives from a paracentric inversion of the long arm of chromosome 7, which results in an in-frame fusion of the N-terminus of the A-kinase anchor protein 9 (*AKAP9*) gene with the C-terminal catalytic domain of *BRAF*. The *AKAP9-BRAF* fusion event results in the loss of the *BRAF* regulatory domains, which exert auto-inhibitory effects on the kinase activity of *BRAF*. The resulting *AKAP9-BRAF* fusion protein shows constitutive kinase activity, and it is able to transmit mitogenic signals to the MAPK pathway and to promote malignant transformation of cells. *BRAF* is thought to play a major role in the carcinogenesis of sporadic PTCs. About 40% of adult PTCs harbor a specific point mutation (Val600Glu) in *BRAF* (1). The Val600Glu *BRAF* mutation is significantly associated with the PTC TCV. Val600Glu mutation constitutively activates *BRAF* by

destabilizing the inactive form of the kinase, thereby shifting the equilibrium toward the active conformation. Therefore, sporadic papillary carcinomas preferentially harbor the *BRAF* mutations rather than chromosomal rearrangements, whereas radiation-induced tumors demonstrate a low prevalence (4%) of *BRAF* point mutations and high prevalence of *RET/PTC* and *AKAP9-BRAF* rearrangements.

Somatic *RET* mutations have been found in 40% to 50% of sporadic MTCs (medullary thyroid cancers). In roughly 75% of cases, medullary thyroid cancer occurs sporadically (1). The mutation at codon 918 is predominant and more aggressive. Somatic mutations at codons 630, 634, 639, 641, 768, 883, and 922 and deletions including codons 630 and 634 have also been described. However, the functional significance of sporadic *RET* mutations in MTC pathogenesis is unclear. The distribution of the Met918Thr in sporadic MTC and its metastasis is nonhomogeneous, occurring in only subpopulations and subsets of multiple metastases, thus implying that the mutation can arise as a secondary event during tumor progression within a metastatic clone or within a single tumor, or that MTC can be of polyclonal origin. See Figures 41-1 and 41-2 for *RET* tyrosine kinase receptor mutations in MEN2 and sporadic MTC.

Adequate testing is required to screen patients at risk for MTC. Consensus was reached at the sixth international workshop on multiple endocrine neoplasia that the decision to perform thyroidectomy in MEN2 should be based predominantly on the result of *RET* mutation testing, rather than on calcitonin (CT) testing. Currently, early genetic screening for *RET* mutations

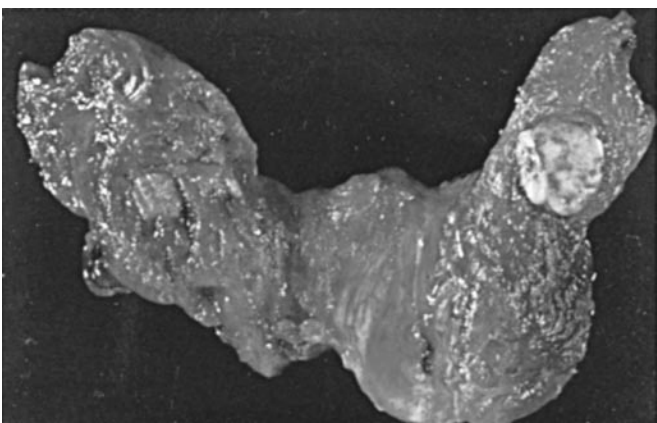


FIGURE 41-2 Bilateral medullary thyroid carcinoma in multiple endocrine neoplasia type 2A. (From Ref. 4, with permission.)

is considered the standard test for MEN2, as patients having a MEN2-specific germ-line mutation have a high risk of developing MTC (5). Because of the critical implications of finding a *RET* mutation, all cases of putatively sporadic MTC or apparently isolated and nonfamilial pheochromocytoma should be also tested for germ-line *RET* mutation. The specifically mutated codon of *RET* correlates with the MEN2 variant, the average age of onset of MTC, and the aggressiveness of MTC. The *RET* codon mutations can be divided into three levels of risk to MTC (6). Children with MEN2B-associated MTC are classified as level 3 (codons 883, 918, 922) or as having the highest risk for aggressive MTC

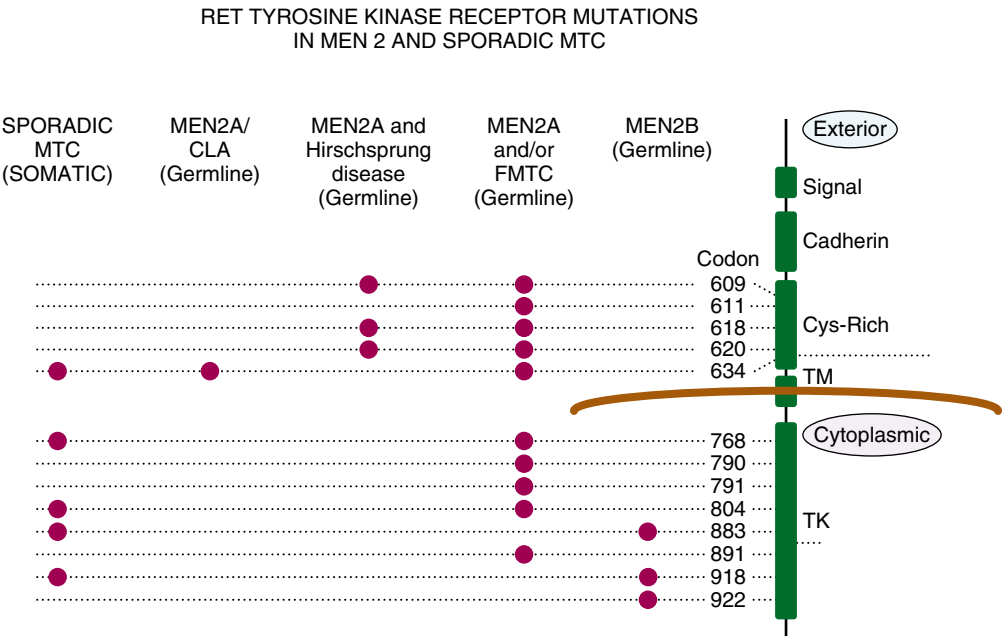


FIGURE 41-1 Molecular abnormalities of the *RET* proto-oncogene in multiple endocrine neoplasia type 2 (MEN2). Mutations of the *RET* proto-oncogene have been identified in MEN2A, familial medullary thyroid carcinoma (FMTC), MEN2A associated with Hirschsprung disease, MEN2A associated with cutaneous lichen amyloidosis (CLA), and as somatic mutations in sporadic MTC. Two regions of the *RET* tyrosine kinase are affected. The first is a cysteine-rich extracellular domain (*Cys-Rich*) important for dimerization of the ret receptor (codons 609, 611, 618, 620, 634). Mutations of individual cysteines at these codons cause *RET* dimerization, activation, autophosphorylation, and transformation. Mutations of the second region, the intracellular tyrosine kinase (*TK*) domain involving codons 768, 790, 791, 804, 883, 891, 918, and 922 cause activation, autophosphorylation, and transformation. A role for the cadherin-like region (*Cadherin*) has not been defined, although it may be involved in an interaction with the glial cell line–derived neurotrophic factor receptor. The most common germ-line mutation is a codon 634 mutation that converts a cysteine to an arginine and accounts for 50% or more of all MEN2 mutations. Somatic mutations of codons 768, 804, and 918 have been identified as somatic MTC. Codon 768 and 804 mutations are rare; a somatic codon 918 mutation is identified in approximately 25% of sporadic MTCs. TM, transmembrane domain. (From Ref. 4, with permission.)

and should have thyroidectomy within the first 6 months and preferably within the first month of life. Thyroid surgery for MEN2B should include a central node dissection (7). Children with RET codon 611, 618, 620, or 634 mutations are classified as having a high risk for MTC (level 2) and should undergo thyroidectomy before the age of 5 years. Children with RET codon 609, 768, 790, 791, 804, and 891 mutations are classified as having the least risk among the three RET codon mutation stratification categories (level 1). They also should have a total thyroidectomy, but no consensus about the recommended age has been reached. The biologic behavior of MTC in patients with the latter mutations is variable, but, in general, MTC grows more slowly and develops at later age than with the high-risk mutations. The guidelines given, of course, should be considered with consideration of the individual clinical context.

Thyroid carcinomas represent a particularly promising paradigm for targeted therapy because some of the key oncogenic events are activating mutations of genes coding for tyrosine kinases, and these occur early in cancer development. The tyrosine kinase inhibitors ST1571 (Gleevec, Imatinib), fenistein, allyl-geldanamycin, and arylidene 2-indolinone (RPI-1) selectively inhibit cell growth and RET tyrosine kinase activity in vitro. Dual inhibition of RET and fibroblast growth factor receptor (FGFR) by using a combination of ST1571 and the FGFR inhibitor PD173074 restrains medullary thyroid cancer cell growth in vitro (8). Two indolocarbazole derivatives, CEP-701 and CEP-751, inhibit RET-MEN2A tumor growth in MTC xenografts. The pyrazolopyrimidine PP1 blocks tumorigenesis induced by RET/PTC oncogenes and induces degradation of activated membrane-bound RET receptors through proteosomal targeting. Another pyrazolopyrimidine, PP2, and the 4-anilinoquinazoline ZD6474 also display a strong inhibitory activity toward constitutively active oncogenic RET kinases. ZD6474 also inhibits angiogenesis and thus exhibits a dual antitumor effect. These small organic compounds show promise for the treatment of human cancers sustaining oncogenic activation of RET and offer the possibility of a successful interventional therapy when conventional pharmacologic and radiotherapeutic regimens have failed. The application of specifically designated combinational therapies, dependent on the unique characteristics of the individual patient and his or her malignancy, may become the standard therapeutic strategy in patients with incurable thyroid tumors.

MEN1

MEN1 is a syndrome complex characterized by hyperparathyroidism with hypercalcemia, neuroendocrine pancreatic tumors (e.g., insulinoma, gastrinoma), and pituitary adenomas that can secrete (singly or in combination) prolactin, growth hormone, and ACTH, to name only a few. The *MEN1* gene is a tumor suppressor gene on chromosome 11q13 and encodes a 610-amino-acid protein termed “menin.” *MEN1* germ-line mutation causes an autosomal dominant pleomorphic multiple neoplasia syndrome called Wermer syndrome. Typically, loss of function of *MEN1* occurs as a single-base DNA change within *MEN1* for

a first hit and a large rearrangement of chromosome 11 for the second hit. Mutations are widely dispersed throughout the gene and only 70% of MEN1 cases have had mutations identified (9). Hyperparathyroidism with resultant hypercalcemia is the most frequent clinical finding (nearly 100% penetrance) and generally is the earliest manifestation of *MEN1* (10). Anterior pituitary tumors, most commonly prolactinomas, occur in approximately 30% of cases, and enteropancreatic tumors in 30% to 80% (Table 41-1). MEN1 is defined as the occurrence of two of these three features. The overall incidence of MEN1 is estimated at 1–2/100,000. Other less common features include adrenal cortical and medullary tumors, thymic and foregut carcinoids, lipomas, facial angiofibromas (Figure 41-3), collagenomas, and leiomyomas. The adrenal cortical tumors may secrete cortisol in excess resulting in Cushing syndrome, and the adrenal medullary tumors may be pheochromocytomas that secrete catecholamines in excess, resulting in hypertension, frequently occurring episodically with headaches and diaphoresis. Zollinger-Ellison syndrome, caused by duodenal gastrinomas, was formerly the major cause of mortality, but with more effective treatment with proton pump inhibitors, malignant enteropancreatic tumors and thymic carcinoids have now emerged as the most frequent causes of mortality in this syndrome. Since enteropancreatic tumors are multifocal and difficult to remove completely, and not all are functional, there is no consensus

Table 41-1 Multiple Endocrine Neoplasia Type 1 Lesions and Their Penetrance

Feature Prevalence
Parathyroid tumor 90% enteropancreatic tumors
Gastrinoma 40%
Insulinoma 10%
Nonfunctioning 20%
Pituitary tumors
Prolactinoma 20%
Other or nonfunctioning 17%
Adrenal tumors
Nonfunctioning 20%
Pheochromocytoma <1% carcinoids
Gastric enterochromaffin-like tumors 10%
Thymic 2%
Bronchial 2%
Facial angiofibromas 85%
Collagenomas 70%
Lipomas 30%
Leiomyomas 10%
Meningiomas 5%
Ependymomas 1%

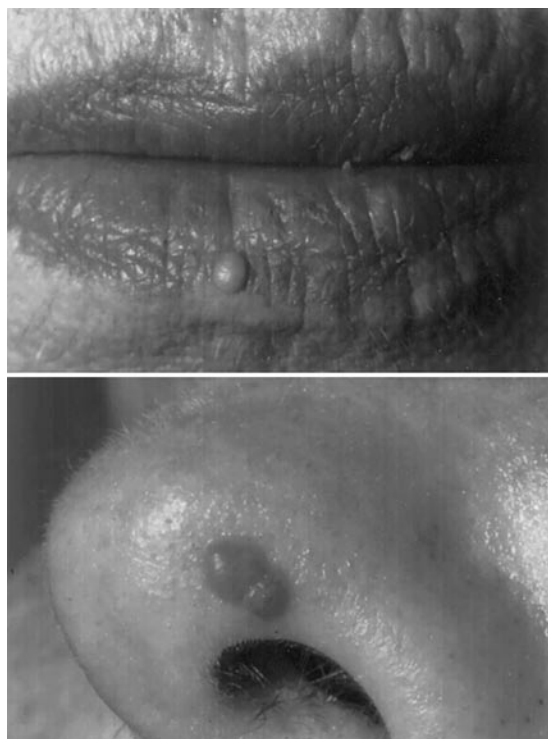


FIGURE 41-3 Facial angiofibroma in patients with multiple endocrine neoplasia type 1. A small, light-pink lesion on the vermilion border of the lip (**top**) and a large, reddish angiofibroma on the nose (**bottom**) are shown. Typical lesions are smaller and may require biopsy for confirmation. (From Ref. 4, with permission.)

on surgical management. Thymic carcinoids, especially in men, may be malignant; hence, prophylactic transcervical thymectomy is advised at the time of initial parathyroidectomy. Within the tumor itself, loss of heterozygosity at 11q13 has been identified in 75% of MEN1–Zollinger-Ellison syndrome carcinoid tumors, in 85% of MEN1 pancreatic endocrine tumors (nongastrinomas), and in 41% of MEN1 gastrinomas (11). However, in contrast to germ-line mutations, *MEN1* mutation testing in tumors is not used in deciding surgical management because it does not have implications for prophylactic surgery (12).

MEN2

MEN2 is a syndrome characterized clinically by hyperparathyroidism with hypercalcemia, medullary thyroid cancer and pheochromocytomas. MEN2, also called Sipple syndrome, is caused by germ-line–activating mutations of *RET* oncogene on chromosome 10, which encodes a transmembrane tyrosine kinase that binds members of the glial-derived neurotrophic factor (GDNF) family by associating with coreceptors for GDNF. Agonist binding promotes receptor dimerization, transphosphorylation of key intracellular tyrosines, binding of intracellular adaptor proteins to phosphorylated tyrosines, and thereby stimulation of ras/mitogen-activated protein kinase and P13 kinase/AKT cascades. Almost all of the germ-line *RET* mutations in MEN2 are missense. MEN2 is considered likely to result entirely from mutations in the *RET* gene, as only rarely has an MEN2 family been identified without

a *RET* mutation. It is estimated that approximately 3% to 5% of MEN2 patients do not have an identifiable *RET* mutation, and it is believed likely that a mutation exists outside the standard exons that has not yet been identified. Oncogenic *RET* mutations show striking and important correlations with the variant of the MEN2 phenotype. MEN2 is divided into three different clinical variants: MEN2A, MEN2B, and familial medullary thyroid cancer, all inherited in an autosomal dominant fashion. The most common phenotypic MEN variant (75%) is termed “MEN2A” this has combinations of thyroid C-cell cancer (i.e., MTC), pheochromocytoma, and hyperparathyroidism. Ninety percent of the *RET* mutations in MEN2A occur in only six cysteines in a small 25–amino-acid domain in the extracellular region with mutation of cysteine 634 on exon 11 as the most frequent cause; others include 609, 611, 618, 620 (exon 10), 630 (exon 11) (13). Rarely, MEN2A may occur in association with Hirschsprung disease (loss of enteric ganglia). The presumptive etiology is that development of enteric ganglia is sensitive to reduced *RET* expression. MEN2B is a distinct variant in which C-cell cancer begins at a much earlier age, and this entity is generally more aggressive clinically than MEN2A, especially in regard the medullary thyroid cancer (14). Almost all *RET* mutations in MEN2B are confined to one cytoplasmic amino acid Met918Thr (95%) or Ala883Phe (<5%; 6). The Met918Thr substitution is frequently a *de novo* mutation located on the allele inherited from the patient’s father. How this particular mutation in *RET* leads to the type 2B phenotype, with MTC, pheochromocytoma, mucosal and intestinal neuromas, and a marfanoid habitus, is not clear, but MEN2B tumors have a different gene expression pattern than do MEN2A tumors. FMTC is characterized by the presence of MTC alone in at least four family members. FMTC mutations are similar to those causing MEN2A, but are more homogeneously distributed among cysteines 609, 618, and 620. Mutations of residues 768, 790, 791 (exon 13), 804, 844 (exon 14), or 891 (exon 15) of the *RET* tyrosine kinase domain have also been found in FMTC patients (12). FMTC is considered the least aggressive of the three MEN2 subtypes. Carrier testing in MEN2 variants has been a model for management of familial medullary thyroid cancer.

Hyperparathyroidism–Jaw Tumor

HPT-JT is caused by germ-line loss-of-function mutations in a gene, *HPRT2*, encoding a protein termed “parafibromin” found on chromosome 1q25–q31. Evidence suggests that human parafibromin occurs in the nuclear paf complex in association with RNA polymerase II, but how its loss leads to tumorigenesis remains to be defined. Hyperparathyroidism caused by multiple, often cystic, and occasionally malignant parathyroid tumors (15%) is the defining and most common feature of HPT-JT (15). Ossifying fibromas of the mandible or maxilla occur in up to 30% of cases, as do renal cysts; more rarely, Wilms tumor, Hurthle cell thyroid adenomas, pancreatic adenocarcinoma, and testicular germ cell tumors are seen. HPT-JT is rare, with only 28 families reported (15), although the exact incidence is probably higher. Regular monitoring is preferable to allow prompt intervention and surgery if and when HPT

recurs. Annual calcium and intact PTH screening, annual renal ultrasound, and appropriate facial radiographs every 3 years for affected kindreds are recommended beginning at age 15 years (16).

Carney Complex

CNC (Carney complex) is a rare disorder with germ-line loss-of-function mutations in the *PRKAR1A* gene within the 17q-linked locus responsible for one form of the disease. The *PRKAR1A* gene encodes the type 1a regulatory subunit of protein kinase A (PKA), which is known to be an important effector molecule in many endocrine signaling pathways. Biochemical assays suggest a net increase in PKA activity following cyclic adenosine monophosphate (cAMP) stimulation. Increased cAMP is mitogenic and may account for the pleiotropic manifestations. *PRKAR1A* seems to function as a classic tumor-suppressor gene in tumors from CNC patients (i.e., mutations of this gene are associated with loss of the normal allele), as shown by loss-of-heterozygosity (LOH) studies, in lesions caused by CNC. On screening a large cohort of 53 CNC kindreds collected over the past 20 years at the National Institutes of Health (NIH) and the Mayo Clinic, mutations of *PRKAR1A* were identified in 15 (44%) of 34 families, as well as seven (35%) of 20 apparently sporadic cases, for an overall mutation rate of 40.7% (17). Another genetic loci has been identified for CNC on chromosome 2p16, but the gene located in this region is still unknown (18). Primary pigmented nodular adrenal disease (PPNAD) is the most common endocrine manifestation, occurring in 25% of affected cases (19). Cortisol hypersecretion may range from the classic picture of Cushing disease to an insidious or cyclical form manifesting first with osteoporosis, myopathy, or cachexia. Pituitary, thyroid (rarely malignant), and testicular Sertoli cell tumors and ovarian cysts also occur (20). Neuroendocrine features include early appearance of pigmented skin lesions, cardiac myxomas, and schwannomas. Surgical removal of hormone-hypersecreting tumors and of cardiac myxomas is the usual treatment. A vigorous search, and then monitoring if not identified initially, for the presence of a cardiac myxoma is important. Testing for *PRKAR1A* mutations is not generally recommended at present for patients with CNC, but may be advised for detection of affected patients in families with known mutations of that gene.

Paranglioma Syndrome

Four forms of hereditary paraganglioma syndrome (PGL (1–4) are recognized. PGL 1, 3, and 4 are caused by germ-line loss-of-function mutations in three of the four subunits of the succinate dehydrogenase (SDH) complex II of the mitochondrial electron transport chain, *SDHD*, *SDHC*, and *SDHB*, respectively, on chromosome 11q23. In PGL, affected subjects typically have multiple head/neck paragangliomas and/or pheochromocytoma, with the latter manifesting clinically with paroxysmal hypertension and catecholamine hypersecretion. The most common tumor site is the carotid body. The basis for tissue-specific tumorigenesis and the basis for the varying clinical expressions of mutations in

different SDH subunits remain unclear, but speculations focus on possible effects on apoptosis pathways and on angiogenesis due to free-radical formation (21). Only four families with *SDHC* mutations have been reported, but 10% of index patients in a large European registry of pheochromocytoma/paraganglioma patients had *SDHB/SDHD* germ-line mutations (9). Among the genotype/phenotype correlations described are a greater incidence of pheochromocytoma and paraganglioma in *SDHD* versus *SDHB* mutation-bearing subjects, when the maternal imprinting of the *SDHD* gene is taken into account (22). That is, individuals inheriting *SDHD* mutations from their mothers do not show tumors since the maternal allele is not expressed. *SDHB* mutations, however, are associated with a greater risk of malignant pheochromocytoma and with occurrence of renal and papillary thyroid cancer. These correlations point to a strategy for prioritizing which gene to sequence first in patients with different clinical presentations. Following appropriate treatment in a multidisciplinary manner, tumor removal with adrenal cortical-sparing laparoscopic surgery (when possible) for pheochromocytoma is the main treatment modality. Despite the potential multiplicity of tumors, the morbidity of total adrenalectomy argues against prophylactic removal of both adrenals for PGL, although this risk must be individually determined on the basis of the clinical context and radiologic studies of both adrenal glands.

von Hippel Lindau

The *VHL* (von Hippel Lindau) gene is a tumor-suppressor gene located on chromosome 3p25–26. *VHL* has an estimated birth incidence of 1/36,000/year and a penetrance of 95% to 100% by age 65. Pheochromocytoma in as many as 20% is the major endocrine manifestation with a mean age of diagnosis of about 18 years, but pancreatic islet cell tumors also occur (23). *VHL* catecholamine tumors are predominantly pheochromocytomas (90%) up to 50% bilateral; sympathetic paragangliomas have also been described. Retinal and cerebellar hemangioblastomas and renal cell cancer (all in up to 60% of cases) are the main neuroendocrine features. The *VHL* gene encodes a subunit of a ubiquitin ligase complex that regulates degradation of the hypoxia-inducible transcription factor (HIF1). Type 2 disease, in which pheochromocytoma is common, is associated with various missense mutations, and is further subdivided into 2A (renal cell cancer rare), 2B (renal cell cancer also), and 2C (pheochromocytoma only). In type 2C, mutated *VHL* protein may still down-regulate HIF1, but abnormal extracellular matrix formation may account in ways unexplained for pheochromocytoma formation. Imaging for diagnosis and surgical treatment can be applied in a more targeted manner on the basis of identification of loss-of-function mutations in the *VHL* gene as the cause of the disease.

Neurofibromatosis Type 1

The *NF1* (neurofibromatosis type 1) gene is a tumor-suppressor gene mapping to chromosome 17q11.2. Patients with *NF1*-associated

pheochromocytoma show loss of the wild-type allele. Neurofibromin, the *NF1* gene product, bears homology to the RAS/GTPase activating protein (GAP). Inactivating mutations in the *NF1* gene are mainly found in the RAS/GAP homology region. Pheochromocytoma in patients with *NF1* occurs at a later age than in MEN2 and VHL disease; the mean age at diagnosis is in the fifth decade. Pheochromocytomas are rare, with frequency estimates of 0.1% to 5.7%. About 22% of *NF1* patients with pheochromocytoma have multiple and/or bilateral tumors (24), although the most common presentation is a single pheochromocytoma. Most are benign, although malignant and sympathetic paragangliomas have been reported. Extra-adrenal pheochromocytomas in patients with *NF1* are rare (about 6%) in contrast to patients with VHL disease (about 30%), MEN2 (about 13%), SDHD and SDHB (about 51%). See Table 41-2 and Figure 41-4 for an outline of hereditary pheochromocytoma/paraganglioma syndromes.

The underlying genetic basis for tumorigenesis of sporadic pheochromocytoma remains to be elucidated (25). Sporadic pheochromocytomas represent the vast majority of tumors with a prevalence as high as 90% (17). In sporadic pheochromocytomas, somatic mutations of the *VHL* gene are uncommon with a prevalence of about 8%. Somatic mutations of *RET* in sporadic pheochromocytomas are also uncommon with about 10% to 20%, the most common being the same mutation found in *MEN2B* (codon 918 somatic *RET* mutation). Somatic mutations in *NF1* have also been described in sporadic pheochromocytomas (24). Somatic and occult germ-line mutations in SDHD have been detected in four of 18 apparently sporadic pheochromocytoma and paraganglioma tumors usually presenting in the late 30s to early 40s (24).

Pituitary Tumors

Despite their relatively common frequency and potential for significant morbidity, the etiology of most pituitary tumors remains unknown. The first candidate in the search for genetic alterations



FIGURE 41-4 A pheochromocytoma set on a background of diffuse adrenomedullary hyperplasia in multiple endocrine neoplasia type 2A. In the normal adrenal gland, the adrenal cortices are separated by a thin (<1mm) band of adrenal medulla (not shown). In this pheochromocytoma there is diffuse expansion of the adrenal medulla. (From Ref. 4, with permission.)

was the G-protein α -stimulating activity polypeptide (GSP). Activating mutations of this protein, which leads to constitutive elevation of adenylyl cyclase activity, have been identified in nearly a third of somatotroph adenomas (25). The presence of this mutation appears to correlate with a densely granulated ultrastructural morphology of somatotroph tumors and possibly with greater GH responsiveness to inhibition by the somatostatin analog octreotide. The identification of the putative tumor-suppressor gene encoding the nuclear protein menin prompted investigation of menin's role in the molecular basis of pituitary tumors. Menin overexpression has been shown to inhibit the activity of the prolactin gene promoter. Prolactin expression among lactotrophic pituitary cells is negatively regulated by activin, a member of the transforming growth factor- β

Table 41-2 Hereditary Pheochromocytoma or Paraganglioma

Syndrome	Gene	Gene Locus	Product	Phenotype
Neurofibromatosis	<i>NF1</i>	17q11.2	Neurofibromin	Pheochromocytoma/sympathetic paraganglioma
von Hippel Lindau	<i>VHL</i>	3p25–26	VHL	Pheochromocytoma/sympathetic paraganglioma
MEN I	<i>MENIN</i>	11q13	Menin	Pheochromocytoma
MEN II	<i>RET</i>	10q11.2	RET	Pheochromocytoma
Paraganglioma 1	<i>SDHD</i>	11q23	Succinate dehydrogenase subunit D	Pheochromocytoma/sympathetic and parasymphathetic paraganglioma
Paraganglioma 2	Unknown	11q13.1	Unknown	Parasympathetic paraganglioma
Paraganglioma 3	<i>SDHC</i>	1q21	Succinate dehydrogenase subunit C	Parasympathetic paraganglioma
Paraganglioma 4	<i>SDHB</i>	1p36.1–35	Succinate dehydrogenase subunit B	Pheochromocytoma/sympathetic and parasymphathetic paraganglioma

(TGF- β) family, an action that is regulated by menin and the Smad pathway. Inhibition of TGF- β signaling appears to follow menin inactivation and is regulated in somatomammotropes via Smad3. In studies on pituitary lactotrope cells, Lacerte et al. reported that menin plays a crucial role in the activin/ TGF- β -induced regulation of prolactin expression and pituitary cell growth (26). Some of these actions involving menin appear to be mediated via activin-induced down-regulation of the pituitary transcription factor, pit-1. Inactivation of menin leads to disruption of activin-induced repression of prolactin expression and pituitary cell growth. The prevalence of pituitary adenomas in patients with MEN1 is approximately 40%. There is no apparent relationship between the site or type of genetic mutation in the *MEN1* gene and the MEN1 disease phenotype expressed. Although genetic analysis has proven that there is a germ-line mutation and loss of the intact allele in tumors associated with the MEN1 syndrome, disappointingly, there is no evidence that mutations of the *MEN1* gene play a significant role in the sporadic pituitary tumors that form the vast majority of these lesions or that down-regulation of this gene is implicated. Nevertheless, a possibility that a component of the encoded menin signaling cascade plays a role in pituitary tumor pathogenesis remains a possibility.

Gastroenteropancreatic Endocrine Tumors

Little is known about the molecular basis of sporadic gastroenteropancreatic endocrine tumors defined as PETs (pancreatic endocrine tumors) and GETs (gastrointestinal endocrine tumors). Functioning PETs induce well-defined hormonal syndromes such as hypoglycemia (insulinoma), Zollinger-Ellison (gastrinoma), a Verner-Morrison (VIPoma), or a glucagonoma syndrome. GETs may become symptomatic by secretion of biologically active peptides, biogenic amines, and tachykinins and may cause characteristic disease such as carcinoid syndrome or Zollinger-Ellison syndrome. In sporadic PETs, chromosomal losses occur more frequently than gains, whereas amplifications are uncommon. These findings point toward a tumor-suppressor pathway and chromosomal instability as important mechanisms associated with tumor progression. Alterations are not randomly distributed on chromosomes but are particularly common in distinct chromosomal regions. Gains are common on 4pq (17%), 5q (25%), 7pq (41%), 9q (28%), 12q (23%), 14q (32%), 17pq (31%), and 20q (27%), whereas genomic losses frequently occur on 1p (21%), 3p (19%), 6q (28%), 10pq (14%), 11q (30%), Y (31%), and X (31%) [20]. Losses of 3p, 6pq, and 10pq and gains of 5q, 12q, and 20q were shown to be associated with malignant behavior, whereas gains of chromosomes 4 and 7 in the presence of losses of 21q are prevailing in metastases (11). Nonfunctioning PETs (NF-PETs), in general, harbor higher numbers of chromosomal gains and losses than functioning tumors. These genetic aberrations occur in chromosomal loci frequently involved in malignant tumors. Gains of chromosome 4 and losses of 6q appear to be early events and are already detectable in 40% and 50% of tumors with a diameter of less than 2 cm. Patients with MEN1 frequently develop PETs. However, somatic

mutations of the *MEN1* gene located on 11q13 are detectable in only 21% of sporadic PETs. Interestingly, the frequency of genomic imbalances and the rate of *MEN1* mutations vary among different types of PETs. Thus, only 7.7% of insulinomas and 8% of NF-PETs exhibit *MEN1* somatic mutations. In contrast, gastrinomas, glucagonomas, and VIPomas show a somatic mutation rate of 37%, 67%, and 44%, respectively. Furthermore, primary pancreatic gastrinomas appear to differ from duodenal gastrinomas: in the former *MEN1* mutations were mainly localized to exon 2, whereas the latter more frequently exhibit mutations in the other exons of the gene. Knowledge about the genetic background of GETs is less advanced than that of PETs. A study by Zhao describes losses of the entire chromosome 18 or of its long arm in 38% of GETs (27). Among GETs, besides gastrinomas, only a few tumors have been examined for somatic *MEN1* gene mutations. The *MEN1* mutation rate in sporadic gastrinomas is high (31%). *MEN1* mutations have also been detected in GETs of the ileum and colon (28). The most frequently known altered gene in GETs is β -catenin. Mutations in exon 3 of this gene, protecting the corresponding protein from phosphorylation and degradation, have been reported in 38% of GETs. This leads to cytoplasmic and nuclear accumulation of β -catenin in 79% of these tumors, indicating other, unknown mechanisms of accumulation of this protein.

Mccune-Albright Syndrome

MAS (McCune-Albright syndrome) is a rare disorder characterized by the association of precocious puberty, polyostotic fibrous dysplasia, and café-au-lait-pigmented skin lesions. Several endocrine disorders, all due to autonomous hormonal hyperproduction, can be associated, such as pituitary adenomas secreting growth hormone, adrenal hyperplasia, or hyperthyroid goiters. Activating postzygotic somatic *GNAS* mutations encode substitutions of Arg201 or Gln227 leading to constitutive, agonist-independent cAMP stimulation by disrupting the intrinsic GTPase activity that normally terminates G-protein activation (29). Sporadic and inherited forms of thyroid and testicular Leydig cell tumors may be caused by somatic and germ-line mutations of the G-protein-coupled thyrotropin and leutinizing hormone receptors. The hallmark of MAS is cell proliferation via constitutive camp activation.

Future Directions

The understanding of the molecular genetics of endocrine neoplasia has had tremendous growth in the past decade and future developments will further investigate germ-line and somatic pathways responsible for tumor pathogenesis. This explosion of knowledge has been particularly useful from a clinical perspective, especially as it pertains to thyroid cancer. Clinical evaluation has begun for a number of targeted therapies, including tyrosine kinase inhibition and angiogenesis inhibition. Other targets include modulators of growth or apoptosis and gene therapy. Each of these targeted approaches holds promise for our future ability to treat patients with disease unresponsive to traditional therapy.

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Gastric and Gastroesophageal Adenocarcinoma

Gastric cancer is an aggressive neoplasm that is associated with an extremely poor prognosis. The median survival for metastatic or unresectable disease is approximately 8 to 10 months. On a global basis, gastric cancer is the third most prevalent malignancy, with an estimated 933,293 new cases in 2002, and the second leading cancer cause of death (nearly 700,000 deaths annually) (1). Almost two thirds of cases occur in developing countries. The incidence of stomach cancer is highest in Japan, Central and South America, and Eastern Asia and much lower in North America and parts of Africa. In the United States, an estimated 22,280 cases of gastric cancer will be diagnosed, and 11,260 patients will die from this disease in 2006 (2). Even within the United States, there is a distinct racial disparity with this disease that is not well understood and is notable for a race-specific propensity for the site of origin of the disease within the stomach, the stage of the cancer at diagnosis, and for survival following diagnosis (3,4). Specifically, in one large cohort study from Southern California, Asian patients were more likely to have localized disease (e.g., lymph node negative; odds ratio [OR], 1.61; 95% CI, 1.23–2.10), less likely to have tumors of the gastroesophageal junction (GEJ) and proximal stomach (OR, 0.22; 95% CI, 0.15–0.31), and less likely to be older than 50 years (OR, 0.58; 95% CI, 0.43–0.77; 3). Perhaps most notable was the marked racial variance in 5-year survival rates in this cohort study: 5-year survival for a patient of Asian ethnicity was 20.9% whereas for white patients, 5-year survival was 10.2%.

The MD Anderson gastric cancer registry demonstrated a similar improved survival rate in Asian patients (5-year survival of 26%), but also notably showed that blacks had the worst prognosis (5-year survival of 9%), and Hispanics and white patients were intermediate (5-year survivals of 13% and 11%, respectively; 4). In this study, the significantly worse survival of black patients was independent of stage, tumor location, or histologic subtype (4). These epidemiology, prevalence, and mortality statistics suggest that cancers of the stomach are of significant clinical relevance to the practicing oncologist and that much of the underpinnings of the disease pathophysiology are not well understood.

Although the understanding of the biology of these diseases is increasing, the development of biologically targeted therapies for the treatment of gastroesophageal cancers has been limited. Cytotoxic therapy remains the standard approach, and although

there is agreement on the active agents and active combination chemotherapy regimens, there is little consensus on *the standard or reference regimen*. This chapter reviews the pathophysiology of gastroesophageal malignancies, highlighting advances in our developing understanding of the biology of this disease.

Lauren's Classification: Diffuse or Intestinal or it Does not Matter?

Virtually all stomach cancers are adenocarcinomas that can be pathologically distinguished according to the Lauren's classification as *intestinal* or *diffuse* pathologic subtypes. Intestinal gastric cancers are generally well differentiated with a glandular appearance and tend to expand through the stomach wall, whereas diffuse gastric cancers are more commonly poorly differentiated and spread as single discohesive cells that infiltrate throughout the stomach wall (Figure 42-1). Intestinal gastric cancers predominate in high-incidence areas, and this histology is responsible for much of the ethnic variation across the globe (5). In contrast, the incidence of diffuse gastric cancer is approximately the same independent of geography or race. It is far more infrequent than intestinal gastric cancer, occurring at an incidence of 0.3 to 1.8 per 100,000 people.

The pathogenesis of intestinal-type gastric cancer follows a multistep progression that is probably initiated by chronic inflammation (e.g., possibly as a result of *Helicobacter pylori* infection, chronic gastritis, or autoimmune gastritis). Intestinal-type gastric cancer progresses through chronic gastritis, intestinal metaplasia, and dysplasia. Alternatively, diffuse-type gastric cancer has no known precursor lesion, although a mutation or epigenetic silencing of the E-cadherin gene (see following sections) appears to be a key carcinogenetic event (6). Although *p53* mutations are identified in approximately 50% of gastric cancers, it may be an early event in intestinal gastric cancer and a late event in diffuse gastric cancer (7). Intestinal gastric cancer is more common in elderly men, whereas diffuse gastric cancer is more common in women younger than age 50 and may have a poorer prognosis.

Despite these differences, the molecular biology distinguishing these two types of gastric cancers is poorly understood. No consistent differences in the molecular pathology of these two gastric adenocarcinoma subtypes have been established. With the

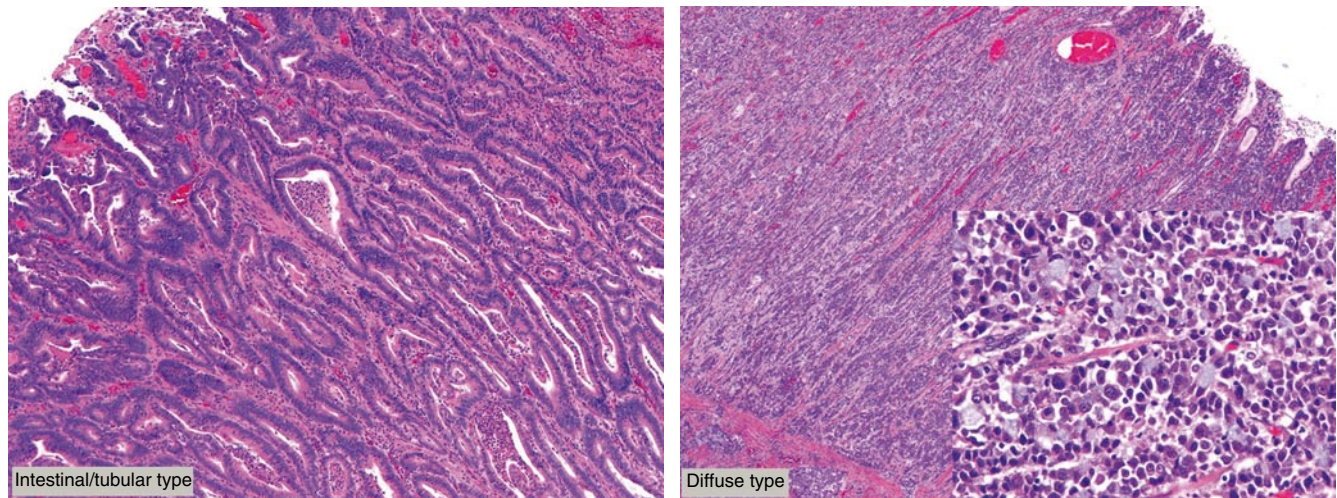


FIGURE 42-1 Lauren's intestinal-type (A) and diffuse-type (B) gastric cancers. Intestinal gastric cancers are generally well differentiated with a glandular appearance whereas diffuse gastric cancers are more commonly poorly differentiated and spread as single discohesive cells that infiltrate throughout the stomach wall.

exception of *p53*, no gene mutation has been identified that occurs regularly in both histologic subtypes. On the other hand, approximately 50% of diffuse gastric cancer histologies demonstrate a mutation in E-cadherin—a mutation that is rarely seen in intestinal-type gastric cancer (see following sections for rare inherited mutations). Chromosomal aberrations and loss of heterozygosity appear to be nonspecific and do not follow any consistent route in the progression of gastric adenocarcinoma of either histologic subtype (8). Clearly, we remain in the infancy of understanding the biologic underpinnings of such obvious histological distinctions.

E. Cadherin and the WNT Pathway: Implications in the Development of Diffuse and Intestinal Gastric Cancer

The Wnt signaling pathway is a central regulatory mechanism of gene expression that is present in vertebrates and invertebrates and is highly conserved in both. It has an essential role in embryonic development but also functions in differentiated cells in a variety of processes including cell cycle regulation. Central to the Wnt signaling pathway is the regulation of β -catenin, an intracellular protein that has multiple cellular functions from cell surface signaling with E-cadherin to nuclear translocation and transcription (Figure 42-2). Mutations in the genes encoding Wnt components are associated with various cancers, including those of the gastrointestinal tract, and in particular, gastric cancer. Diffuse gastric cancer is associated with loss of E-cadherin function in approximately 50% of cases (9). Germ-line mutations in E-cadherin (*CDH1*) are associated with loss of E-cadherin function and the familial form of diffuse gastric cancer, hereditary diffuse gastric cancer (10). Because E-cadherins are components of adherens junctions, this observation is consistent with the loose cell–cell attachment characteristic of the histology of diffuse type gastric tumors. Also, because the E-cadherin/ β -catenin complex normally sequesters a fraction of the total complement of β -catenin in the intracellular

membrane compartment, loss of membrane-bound E-cadherin is expected to result in an increase in the cytoplasmic and nuclear pools of β -catenin. This has been demonstrated in a number of studies, in which increased nuclear localization of β -catenin was observed in diffuse gastric cancer that also demonstrated loss of membrane-bound E-cadherin immunostaining (11).

In contrast to diffuse gastric cancer, intestinal gastric cancer is known as the “epidemic-type” of gastric cancer because the high risk areas of gastric cancer across the globe are due to the high incidence of the intestinal histopathologic phenotype of gastric cancer in those areas. There is a notable defined pathologic sequential carcinogenesis of intestinal stomach cancers beginning with multifocal atrophic gastritis followed by intestinal metaplasia, dysplasia, and then carcinoma (12). The Wnt signaling pathway is also implicated in the development of intestinal gastric cancer. Although decreased E-cadherin expression is associated with diffuse gastric cancer, increased cytosolic concentrations of β -catenin and its nuclear translocation appear to be associated with the development of intestinal gastric cancer (13). Specifically, *APC* gene mutations and mutations in the third exon of β -catenin lead to decreased phosphorylation of β -catenin and resultant reduced proteolytic degradation of this protein. This results in cytosolic accumulation of β -catenin, nuclear translocation, and malignant transformation. Indeed, Ebert and colleagues confirmed these findings with the identification of mutations in the *APC* gene and in the third exon of β -catenin leading to increased β -catenin cytosolic levels and nuclear localization in intestinal gastric cancer (13,14). Somatic mutations in *APC* genes in gastric tumors have also been reported by others (15,16) and are believed to occur in approximately 30% of intestinal type gastric cancers (17,18). Persons with a germ-line mutation of the *APC* tumor-suppressor gene are found to have a tenfold increased risk of developing gastric cancer as compared with healthy persons (19). Somatic mutations in β -catenin have also been observed as well, although its reported incidence has varied widely from as low as 1% to 2% in German and Chinese populations (20,21) to 27% in a Korean

of developing a noncardia gastric cancer is 5.9-fold higher with *H. pylori* infection and that *H. pylori* did not seem to increase the risk of gastric cardia tumors (hazard ratio, 1.0; 95% CI, 0.7–1.4).

H. pylori infection causes release of reactive oxygen species, mitogenic stimulation, and constitutive inflammatory response in the gastric epithelium (26). Two bacterial genes, *cag A* and *Vac A*, are the known determinants of virulence. The *cag A* gene, also known as the cytotoxin-associated gene-A, is a marker for a “pathogenicity island” composed of approximately 20 genes that increase the turnover of gastric epithelium by delivering *cag A* protein into the epithelial cells. The *cag A*-positive *H. pylori* strains are associated with a greater risk of intestinal noncardia gastric adenocarcinoma compared with *cag A*-negative strains (27).

Nuclear factor- κ B (NF- κ B) is implicated as the mediator of *H. pylori*-associated gastritis in patient samples (28). In one study of 41 patients with *H. pylori*-associated gastritis, nuclear activation of NF- κ B was significantly increased in the setting of concurrent infection with *H. pylori* compared with uninfected individuals. The nuclear staining for NF- κ B was associated with infiltrative inflammatory cells, including vascular endothelial cells, macrophages, and B-lymphocytes within the lamina propria.

NF- κ B expression was also examined in 64 human gastric tumor samples by immunohistochemistry (29). In this analysis, nuclear and cytoplasmic NF- κ B expression profiles were shown to be significantly greater in malignant gastric tissue than adjacent normal tissue pairs ($p < 0.0001$). Furthermore, a high expression of NF- κ B in gastric cancer was a significant poor prognostic factor, specifically significantly associated with several aggressive phenotypic characteristics including lymphatic invasion, increased tumor size, worse pathologic stage, and peritoneal metastases. In that study, the 25 patients with high NF- κ B nuclear staining (e.g., $>25\%$) had an approximate 20% 4-year survival versus 75% 4-year survival in the low NF- κ B group ($p = 0.02$).

Altogether, *H. pylori* are implicated in the development of noncardia gastric cancers. Certain *H. pylori* strains may carry a higher risk of developing the disease. Activation of the NF- κ B pathway is implicated as a possible key mediator of carcinogenesis. It is overexpressed and activated constitutively in cell culture and in patient samples when compared to adjacent normal tissue by immunohistochemistry (IHC). In cell culture, reduction of NF- κ B activity is associated with a significant antitumor effect. Finally, NF- κ B appears to be not only an important anti-apoptotic transcription factor, but also a prognostic factor for survival in gastric cancer.

A long-term prospective case control study was performed to examine the role of *H. pylori* infection in the development of gastric cardia and gastroesophageal junction adenocarcinoma (30). The study of Finnish males who participated in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study in Southern Finland included 29,133 eligible Finnish male smokers between the ages of 50 and 69 between the years 1985 and 1988. Among this population, 243 patients were diagnosed with gastric adenocarcinoma through April 30, 1999, of whom 234 (96%) had adequate baseline serum samples for *H. pylori* analysis. The investigators selected an equal number of control subjects who were matched for age from the ATBC study who remained cancer free through the follow-up period. Gastric cardia and GEJ adenocarcinomas were

identified in 61 cases (26%). As expected and in support of earlier studies, *H. pylori* seropositivity was more common in noncardia gastric cancer patients than in the matched control subjects (86% vs. 71%; adjusted OR, 3.3; 95% CI, 1.72–6.42). However, in contrast, *H. pylori* seropositivity was less common in patients with cardia and GEJ adenocarcinoma than in matched control subjects (57% vs. 72%; adjusted OR, 0.28; 95% CI, 0.09–0.86; 30). Thus, these investigators observed opposing associations between *H. pylori* seropositivity and either noncardia or cardia gastric cancers, suggesting that the presence of *H. pylori* may be somewhat protective of developing a cardia or GEJ adenocarcinoma.

The inverse association of *H. pylori* with gastric cardia and GEJ adenocarcinoma was seen in other studies on Western populations (31). This inverse association implies that the substantial increase in the incidence of GEJ adenocarcinoma observed in Western populations may possibly be due to the reduced occurrence of *H. pylori* infection in the same population. The molecular rationale for this inverse association remains speculative at present.

Gastric Cancer and Bone Marrow-Derived Stem Cells

One concept of the pathogenesis of *H. pylori*-associated gastric cancer is that of developing a chronic infection within the lining of the stomach leading to increased cell turnover and eventually to malignancy. Interestingly, the same inflammatory environment that favors the development of malignancy has also been linked to homing and engraftment in peripheral tissue of bone marrow derived stem cells. Exploiting this concept in the mouse equivalent of *H. pylori*-induced gastric cancer, Houghton and colleagues demonstrated that bone marrow-derived stem cells populate proliferative zones within the stomach in the setting of chronic *Helicobacter* infection (32). Furthermore, in time, these transplanted bone marrow-derived cells were responsible for the development of intraepithelial neoplasia seen in the 1-year-old infected mice, thus strongly implicating bone marrow-derived stem cells as the cell responsible for the development of gastric cancer in this model (32).

These findings have significant implications in the understanding of the development of the disease. In particular, this model adds to the multistep model of the development of gastric cancer beginning with gastritis and evolving to metaplasia, dysplasia, and finally carcinoma (Figure 42-3). Specifically, in the setting of chronic inflammation, bone marrow-derived stem cells are recruited to populate the ulcerated/ injured tissue as part of normal healing. However, in this setting, the bone marrow-derived stem cells fail to differentiate properly and instead progress to metaplasia, dysplasia, and cancer. Additionally, the model also supports the purported role of the immune system in the development of gastric cancer. Specifically, familial clusters of gastric cancer have been identified in families with polymorphisms within proinflammatory genes including interleukin 1- β and TNF- α (33). Polymorphisms in these genes can result in elevated cytokine levels resulting in a 27-fold increase in risk of developing gastric cancer in patients also infected with *H. pylori* whereas there appears to be no increase in risk in the absence of infection.

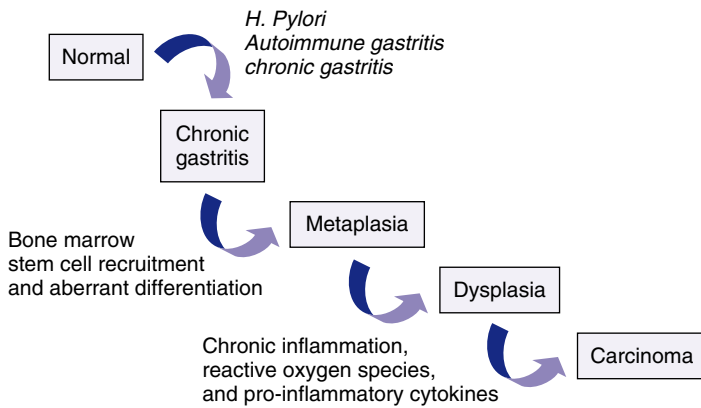


FIGURE 42-3 Multistep model of gastric cancer progression as proposed by Correa and the emerging role of bone marrow–derived stem cells modified from Ref.49, with permission.

The prospect of a cancer stem cell has significant therapeutic implications (reviewed by Miller et al. [34]). For example, in the context of cancer stem cells differentiating into aberrant differentiation pathways, the process of acquiring increasingly malignant features within preexisting closely related but different malignant clones results in tumor heterogeneity, and by selection, chemotherapy resistance. In addition, cancer stem cells may be inherently more resistant to DNA damaging therapy due to reduced turnover and DNA synthesis. These features may explain in part the limited success of traditional cytotoxic therapies.

RUNX3 and Gastric Cancer: A New Tumor Suppressor?

Three mammalian Runt-related (RUNX) genes, *RUNX1*, *-2*, *-3*, encode a set of closely related DNA-binding proteins. These three genes comprising the RUNX family of transcription factor proteins share a high degree of sequence homology, and in particular, each member's amino terminal shares a highly conserved 128–amino acid sequence highly homologous to runt, one of the *Drosophila* pair-rule gene products (35). RUNX genes encode α subunits (polyomavirus enhancer-binding protein 2 α /core-binding factor- α , PEBP2 α /CBF α). These subunits combine with β subunits (PEBP2 β /CBF β) to form the heterodimeric transcription factor, which was initially discovered as PEBP2, or CBF (36). Each member of the RUNX family of transcription factors plays important roles in normal developmental processes as well as in cancers. *RUNX1* is also known as AML1, and plays a vital role in hematopoietic development. Ablation of *RUNX1* or CBF β results in lethality and a complete lack of fetal liver hematopoiesis. *RUNX1* is an important translocation breakpoint in the development of human leukemia, in particular the TEL-AML1 t(12;21) fusion accounts for 20% of acute lymphoblastic leukemia and the AML1-ETO t(8;21) fusion accounts for 12% of acute myeloid leukemias (37). *RUNX2* is critical for osteoblast differentiation and bone formation, and its overexpression in transgenic mice predisposes to T-cell lymphomas (38).

Recently, *RUNX3* has been implicated in gastric carcinogenesis (39). *RUNX3* is strongly expressed in gastrointestinal organs in the developing embryo and throughout adult life of the mouse. The gastric epithelium in *RUNX3* knock-outs displays

hyperplasia and a reduced apoptotic rate. Primary cultures of *RUNX3*^{-/-} gastric epithelial cells are less sensitive to transforming growth factor- β (TGF- β), due in part to failure to induce apoptosis. These characteristics led investigators to question the role of *RUNX3* as a tumor suppressor. In this regard, Li and colleagues analyzed *RUNX3* expression in a series of gastric cancer cell lines and primary human gastric tumors. *RUNX3* expression was reduced or lost in 60% of the primary human tumors analyzed, rising to nearly 90% loss in tumors of patients with advanced-stage gastric cancer (39). Unfortunately, the mice do not form gastric cancers, due to early death (by day 10), possibly due to starvation. However, Li and colleagues did demonstrate the malignant potential of *RUNX3* loss by a xenograft mouse model. Specifically, they crossed *RUNX3*^{+/+} and *RUNX3*^{-/-} mice with *p53*^{-/-} mice, and obtained cell lines from epithelial cells of the glandular stomach from each. The reason for using *p53*^{-/-} mice was to enhance the chance of generating cell lines. They showed that cell lines obtained from *RUNX3*^{-/-}*p53*^{-/-} mice grew faster on monolayer cultures than those from *RUNX3*^{+/+}*p53*^{-/-} mice. Perhaps most intriguingly, *RUNX3*^{-/-} cells induced adenocarcinomas when transplanted into nude mice, whereas the *RUNX3*^{+/+} cells did not (39).

These data suggest that *RUNX3* may indeed be an important tumor suppressor oncogene in the development of gastric cancer.

Molecular Targets in Development for Gastroesophageal Cancer Treatments

One of the primary goals of understanding the molecular biology of gastric and gastroesophageal cancers is to develop targeted therapies specific to the disease. However, targeted agents examined in upper gastrointestinal malignancies have been first demonstrated to be useful in other tumor types.

The inhibition of tumor angiogenesis represents a new therapeutic strategy in the treatment of solid tumors. Vascular endothelial growth factor (VEGF) is a dimeric, heparin-binding glycoprotein that functions as a potent mitogen for vascular endothelial cells, promoting their migration and organization for the neovascularization of micrometastases. VEGF is expressed in gastric cancer, and its expression increases with increasing stage and tumor burden (40). VEGF expression is a negative prognostic factor for survival in patients with gastric cancer. VEGF expression

or serum concentration has been positively correlated with vascular involvement and lymph node, liver, and peritoneal metastases (41). Inhibition of VEGF activity by an immunoneutralizing antibody, both when administered as a single agent and when administered together with chemotherapy, demonstrated antitumor efficacy in a gastric carcinoma xenograft model (42). These data suggest that VEGF and angiogenesis appear to play an important role in the pathogenesis and progression of gastric carcinoma, and its inhibition may be of therapeutic value.

Bevacizumab (Avastin; Genentech Inc. San Francisco, CA) is a recombinant, humanized monoclonal antibody that targets VEGF. When bevacizumab was administered in combination with chemotherapy, significant improvements in antitumor efficacy were observed in several malignancies, including colon, lung, and breast cancer; the drug is approved by the U.S. Food and Drug Administration (FDA) as first-line treatment when combined with chemotherapy for metastatic colorectal cancer. Bevacizumab has been evaluated in combination with irinotecan and cisplatin in gastric and GEJ cancers in a multicenter phase 2 study (43). Forty-seven patients with previously untreated metastatic gastric/GEJ adenocarcinoma were treated with bevacizumab 15 mg/kg on day 1 and irinotecan 65 mg/m² and cisplatin 30 mg/m² on days 1 and 8, every 21 days. The primary endpoint was to demonstrate a 50% improvement in time to progression over historical controls, which were estimated to be about 5 months with irinotecan and cisplatin alone, and with the aim to improve time to progression (TTP) to approximately 7.5 months. With a median follow-up of 12.2 months, median TTP is improved to 8.3 months, and median survival improved to 12.3 months. Although there was no apparent increase in chemotherapy-related toxicity, including nausea, vomiting, diarrhea, and myelosuppression, there was a significant portion of patients with significant hypertension, two patients with a gastric perforation, and one patient with "near" perforation (6%). This study demonstrates that the addition of bevacizumab to chemotherapy in the treatment of gastric and gastroesophageal adenocarcinoma is associated with improved efficacy. What remains uncertain is whether the improved efficacy will be justified if the rate of perforation (i.e., 4%–6%) demonstrated in this clinical trial is in fact a reality. Certainly, additional evaluation of anti-angiogenic agents is warranted (43).

The epidermal growth factor pathway has also been implicated in the pathogenesis of upper gastrointestinal malignancies (44,45). The EGF receptor inhibitor (Erb-B1), gefitinib (ZD1839,

Iressa) was examined as salvage therapy in 75 patients with gastric and GEJ tumors (46). Minimal antitumor activity was observed with one patient achieving a partial response and 12 with disease stabilization. Notably, in 32 patients who underwent serial biopsies, the phosphorylation status of the EGF receptor was significantly reduced with gefitinib, but the inhibition of proliferation (in an ex vivo assay) was more dependent on levels of phosphorylated Akt (47), suggesting that resistance to EGF receptor inhibitors may be mediated downstream through the PI3-Akt pathway. In another study, another oral tyrosine kinase EGR receptor inhibitor, erlotinib, was examined in the first-line setting in gastric and GEJ tumors (48). In this study, inhibition of the EGF receptor pathway appeared to have greater efficacy in tumors of the GEJ than gastric tumors, suggesting perhaps differences in the molecular biology of these tumors. The investigators, however, did not report the Lauren class (e.g., diffuse or intestinal) in their analysis.

Future Directions

Gastroesophageal cancers are the most common cause of cancer-related mortality worldwide. Palliative chemotherapy is considered for most patients with these diseases, with benefits in quality of life and in survival. Despite our advances in chemotherapy treatments, survival for metastatic disease remains under 1 year. Clearly, the genetic determinants of the development of the disease are just now beginning to be better understood. With the explosion of new biologically directed therapies for the treatment of a variety of solid tumors in the past decade, the future of the treatment of this disease will clearly lie in our better understanding of the pathogenesis of the disease and in our better understanding of how to exploit the molecular biology of the disease to our advantage. We are still struggling with defining a standard cytotoxic combination for the treatment of this disease. Understanding who, when, and how to administer future targeted therapies with *standard* cytotoxic therapies will undoubtedly have a major impact for the future treatments of this disease. The development of targeted therapies in gastroesophageal malignancies that are highly effective in their own right (e.g., imatinib in gastrointestinal stromal tumors) requires a better understanding of the molecular biology and pathogenesis of these diseases and the identification of more specific targets. Herein lies the future of the treatment of gastroesophageal malignancies.

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From Bench to Bedside with Targeted Therapies

Molecularly Targeted Therapy and Personalized Medicine

Cancer therapy, which encompasses the prevention of premalignant or early cancer (chemoprevention) and the therapy of localized or advanced cancer, is arguably at the most exciting time in its history. It is at the confluence of two new movements, one toward personalized medicine and the other toward the use of new molecularly targeted cancer therapeutics that exploit the tumor's genetic and molecular signature. These movements provide many challenges, but also the opportunity for making paradigm shifts in the way we think of and treat cancer, for dramatically reducing the side effects of cancer therapy, and for increasing long-term stabilization and cure of the disease.

Molecularly targeted therapy uses an agent (or combination of agents) that acts with a high degree of specificity on a well-defined target or biologic pathway that drives the cancer phenotype, so that when the patient is treated with the agent(s), there is destruction of the cancer cells, with minimal harm to normal cells. Genomic, proteomic and other "omic" analyses have taken us to the point where we are able to identify literally hundreds of abnormalities in gene expression in some way related to cancer. However, not all these changes cause or even contribute to the cancer phenotype. Some, probably many, are secondary and due to the abnormal environment the cancer cell finds itself in within the tumor. The question is, then, what makes a good molecular target for cancer therapy? Clearly it should be essential to an aberrant processes in cancer, ideally one of the hallmarks of cancer described by Hanahan and Weinberg (1), namely, self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. The target should also show minimal redundancy, meaning that its inhibition gives a major change in the cancer phenotype being measured, without other pathways taking over. It is certainly advisable to have demonstrated these characteristics before embarking on an extensive drug discovery effort. The evidence might take the form of the inhibition of the target by knock-down, by homologous recombination, by antisense, or, now, by small-interference RNA, always taking into account the limitations of the technique.

However, drugs themselves are rarely selective enough for proof-of-principle validation of a particular target. A cancer drug need not have just one target. No drug is ever completely selective, and the other targets may contribute to the drug's therapeutic effects. If the other targets are not cancer related they may be a cause of toxicity. Targeted therapies clearly need to be administered to patients whose cancer expresses the target, and it is therefore necessary to define the patient population to be treated. The target level in the cancer should be measurable, and should correlate with some recognized patient outcome such as quality of life, time to progression, patient morbidity, or survival.

Personalized medicine or individualized treatment is the model of the way medicine will evolve through the use of specific treatments and therapeutics best suited for an individual's genotype. There has been much discussion of the scientific, social, economic, and ethical considerations of personalized medicine (2). Cancer therapy presents a unique example of personalized medicine where not only is the individual's genotype important, in so far as it determines the therapeutic and toxic response to a drug (the pharmacogenotype), but the genotype of the cancer cell is also important since it determines the response to therapy.

Achieving Personalized Medicine

To achieve the goal of personalized medicine it is necessary not only to have agents with defined molecular specificities, but to have minimally invasive biomarker and imaging tests that will identify which patients have the target in their tumor and the patient's pharmacogenotype. A related objective is to have a way to measure the effect of a drug on its molecular target in the tumor to be able to answer important questions, such as how much drug is required to inhibit the target in a tumor and is there is a benefit to giving more drug or will this only increase toxicity (3)? It is also desirable to have a test to assess early response so that nonresponding patients can be spared unnecessary treatment and be moved to alternate therapies.

A limitation to the goal of achieving personalized medicine is our incomplete knowledge of tumor biology and the target-related effects of the drug in normal tissues. For example many protein kinase-inhibitory cancer drugs inhibit other kinases, which may

lead to additional therapeutic opportunities, but can also lead to unexpected toxicities (4). These drugs are sometimes referred to euphemistically as pan-inhibitors or less endearingly “dirty” drugs. We also face the challenge of identifying off-target toxicities that can limit or even curtail a drug’s use. The example of the unexpected cardiac toxicity of high doses of the COX-2 inhibitors over prolonged periods is an example (5).

There is widespread frustration with the cost and time it takes to bring a new cancer drug to clinical use, typically it is claimed more than \$800 million to develop a drug. After research, which may take many years, has identified a candidate target and drug, further preclinical studies typically require 3 years followed by up to 7 years from investigational new drug (IND) filing until approval (6,7). There are many ways that clinical trials of molecularly targeted cancer drugs could be hastened. Frequently when a drug first enters clinical trial, there is no validated biomarker or imaging test to select patients who are most likely to respond or for identifying the molecular response to treatment. Validated in this context means that the test is standardized and has been shown to be reproducible across institutions, if not in patients, then at least in animal models. Often a biomarker or imaging test may become available only after the drug has been in trial for some time. An example is the lack of a validated biomarker for the epidermal growth factor receptor (EGFR) inhibitor gefitinib (Iressa), which was given early approval by the U.S. Food and Drug Administration (FDA) for refractory non-small cell lung cancer but ultimately failed to demonstrate prolonged patient survival (8). It is now known that there is a subset of lung cancer patients with an activating mutation of the EGFR kinase that confers sensitivity to gefitinib and other similar agents (4). It is a moot point whether knowledge of this mutation would have altered the course of development of gefitinib and its ultimate fate. An example where subsetting of patients has been of benefit in gaining regulatory approval and in application to clinical practice is the use of a her2/neu test for women with breast cancer receiving the anti-her2/neu monoclonal antibody trastuzumab (Herceptin). Even in this case, the presence of high receptor levels is predictive of sensitivity to therapy in only a third of cases.

The requirement for disease-specific drug trials means that responding patients still have to be identified by a separate trial in each disease group. Since molecular targets are frequently found in multiple types of cancer a move to target-specific rather than disease-specific clinical drug trials may be beneficial. However, this will require a paradigm shift from the way cancer drugs are developed. The traditional disease-specific trials treating large numbers of patients who are likely to fail on a drug can be criticized ethically if there is knowledge, or knowledge could be obtained, that would allow treating smaller groups of patients, perhaps across cancer diseases types, who have the molecular target and thus a chance of response. Unfortunately the presence of the target molecule in a cancer is not always a guarantee that the malignant cells are sensitive to an agent against that target, as noted with HER2 and trastuzumab.

A summary of the new technologies for cancer therapy development and the challenges for realizing personalized medicine are shown in Figure 43-1.

Opportunities for New Drug Development

Technology Advances

The search for molecularly targeted therapies is being aided, and in some cases driven, by paradigm shifting new technologies, many of which are discussed within the third edition of the *Molecular Basis of Cancer* and are summarized in Figure 43-1. The new technologies include the “omic” technologies, genomic profiling to measure all mRNA transcripts, proteomics, glycomics, lipidomics, and metabolomics. Additional exciting new technologies that have become available are nanobiotechnology, microfluidics, genome-wide gene functional profiling using interference RNAs, and computational biology for screening, drug design, mechanistic simulations, and bioinformatics.

Nanobiotechnology

Nanotechnology is the creation and utilization of materials and devices on the nanometer scale at the level of atoms, molecules, and supramolecules, and nanobiotechnology is its application to life sciences, including molecular diagnostics, drug discovery, drug delivery, and the development of nanomedicine (9,10). Some of the potential applications of nanobiotechnology in cancer medicine are given in Table 43-1. The greatest immediate impact of nanobiotechnology is likely to be on the molecular diagnostics of cancer, sometimes referred to as nanodiagnostics (11). For example, quantum dots, highly fluorescent nanocrystals, or gold nanoparticles may be used to label cancer cell-homing antibodies for diagnostic imaging. Nanotechnology-based methods are also being developed for cancer drug delivery (12). Nanotechnology platforms in the form of microfluidics or the “lab on a chip” are providing ways for high-throughput, sensitive functional assays for target identification and drug discovery (13).

Functional Genomic Screening

Interference RNA (RNAi) allows the targeted post-transcriptional degradation of messenger RNA thereby inhibiting the synthesis of the specific protein and effectively leading to silencing of gene expression. The effectors are RNAi duplexes of approximately 21 to 23 nucleotides that are key intermediaries in the specific degradation of target mRNA following incorporation into the RNA-induced silencing complex (RISC) in the cytosol. Genome-wide RNAi functional screening (gene screening) is now a reality, relying on different technologies such as long double-stranded (ds) RNAs, in vitro diced short-interfering (si) RNAs, synthetic siRNAs, and short-hairpin (sh) RNAs, whereas advances in screening technologies and data analysis allow the adaptation of screening methods to probe more complex cellular processes (14). Some of these molecules are being explored as therapeutic agents once appropriate targets are identified (15). A related and rapidly advancing field is that of micro-RNA (miRNA), a class of small, non-coding RNAs first described less than 4 years ago in *Caenorhabditis elegans* and now with several thousand members in multiple organisms (16). miRNAs are naturally occurring, single-stranded RNAs that regulate the expression of other genes. They are processed in the cytoplasm

NEW TECHNOLOGIES FOR PERSONALIZED MEDICINE

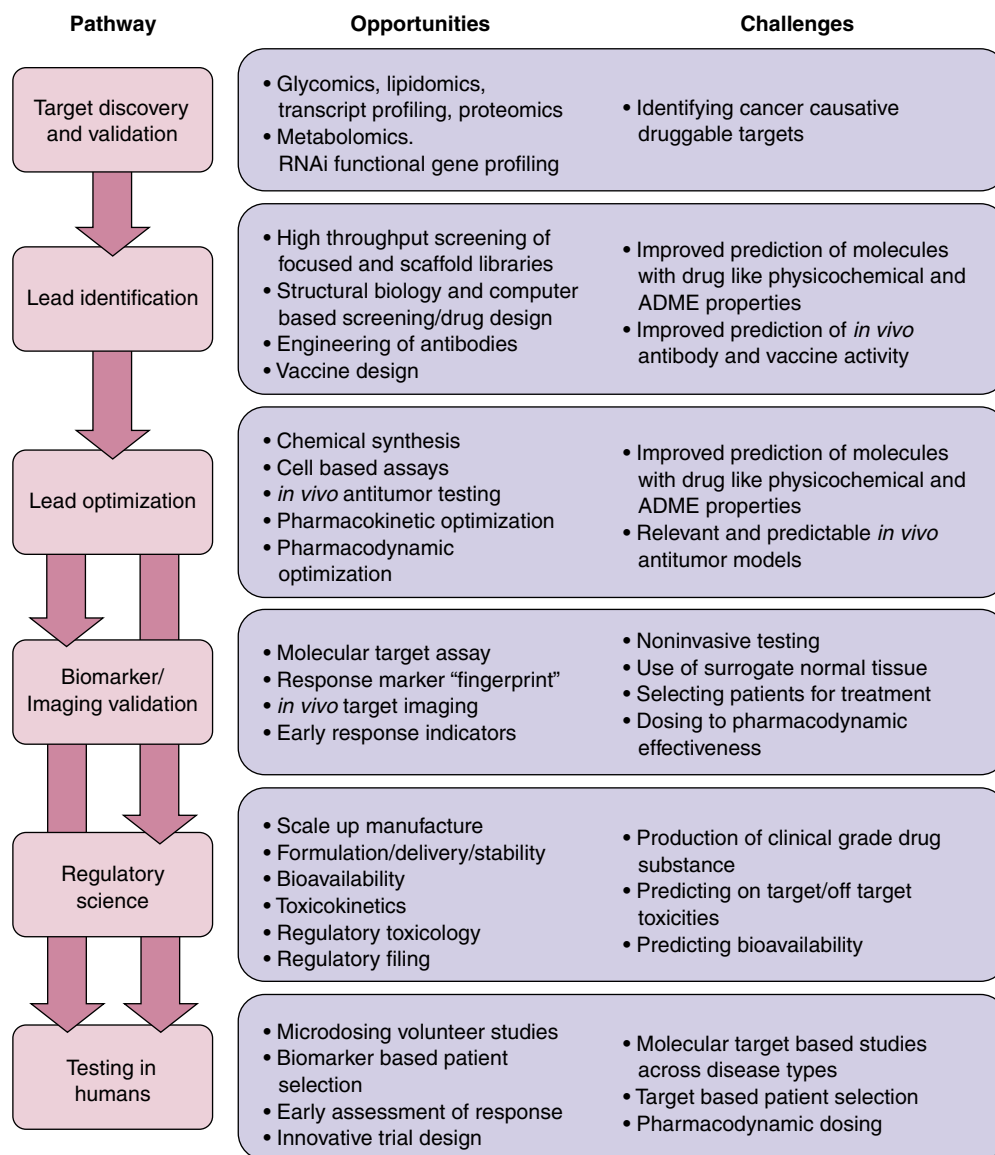


FIGURE 43-1 NEW APPROACHES AND CHALLENGES FOR PERSONALIZED CANCER MEDICINE. Therapy development is shown as linear process whereas in reality it is iterative in all its stages. A full description can be found in the text.

from primary transcripts to short stem-loop structures, and ultimately functional miRNAs complimentary to regions in the 3' untranslated regions (UTRs) of one or more messenger RNAs, which they target for degradation, or they may block protein translation. miRNAs are frequently found at hot spots for chromosomal abnormalities in cancer and are involved in the coordination of cell proliferation, cell death, differentiation, and stress resistance. miRNA expression profiles can be used to classify cancers on the basis of developmental lineage and differentiation whereas miRNAs with anti-apoptotic and prosurvival properties offer novel therapeutic targets.

Computational Cancer Biology

Computational biology encompasses the simulation of known biology (systems biology) and the inferential mining of data

(bioinformatics) (Table 43-2; 17). Only through the use of advanced computational techniques and mechanistic dynamic simulations of known biology can we hope to understand the complexities of signaling mechanisms within normal and cancer cells, beyond what we can determine by experiment or intuition (17,18). However, computational modeling is limited by the lack of high-quality quantitative data from studies designed to answer specific questions. Further the analysis must be iterative with computational models being developed from high-quality data and predictions from the models being tested by well-designed interventional studies. The data from these studies will be needed to constrain and further develop the models. Bioinformatics is essential for the organization and inferential data mining to extract patterns and relationships from the vast amounts of information generated by the explosive growth of the omic technologies. The combination

Table 43-1 Uses of Nanotechnology in Cancer Medicine

Technology	Application
Nanovectors	
Lipid based (liposomes)	Drug delivery
Polymer based (self assembling dedrimers)	Drug delivery, imaging
Porosified silicon	Biodegradable drug delivery
Nanoshells gold over silica	Localized thermal ablation
Nanochannels	
Passive release	Drug delivery
Active release	Drug delivery
Contrast agents	
Gadolinium, ferric oxide	Magnetic resonance imaging
Lipid encapsulated microbubbles	Ultrasound
Nanodiagnostics	
Quantum dots	Optical imaging
Semi-conductor nanocrystals	Optical imaging
Gold nanoparticles	Optical imaging
Nanoarrays	
Nucleic acids, proteins	Omic profiling
Biomolecular sensors (nanocantilever arrays)	
Multiplexed DNA	Gene mutation
Silicon nanowire biotransistors	Proteomics
Carbon nanotube biotransistors	Proteomics
Microfluidics	
Precise movement of nanoliter amounts of fluid	“Lab on a chip”

Source: Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nature Rev Cancer* 2005;5:161–171, with permission.

of systems biology and bioinformatics, for example in a whole-cell model of pathways and molecular constituents linked to cellular phenotype (19) with omic data when the cell is perturbed offers the power, through in silico testing of therapeutic agents in different genetic backgrounds and in different combinations, for optimization of individual therapy, thus, realizing the goal of personalized medicine. The computer-based in silico screening of virtual chemical libraries for docking to known protein structures will greatly aid the identification of lead compounds for drug development, enabling rational drug design (20,21).

Table 43-2 Computational Biology for Personalized Cancer Medicine
Mechanistic Dynamical Simulations of Known Biology
(Systems Biology)

Cell cycle control
Egfr/her2 interactions
Chromosomal aberrations
Tumor/microenvironment
Metastasis
Protein/protein interactions
Inferential data mining (bioinformatics)
Extracting patterns and relationships from high throughput data
Bayesian probabilistic models to determine cellular networks
Drug discovery
<i>In silico</i> high-throughput docking
Structure guided design

Source: Khalil IG, Hill C. Systems biology for cancer. *Curr Opin Oncol* 2005;17:44–48, with permission.

Targets

The search for new cancer-relevant targets continues unabated. Almost 300 mutated genes have been causally implicated in human cancer (22,23). However, this does not mean this is the total number of cancer drug targets. The products of genes that that modulate, or are modulated by the mutated genes, tumor-suppressor gene pathways, epigenetic regulatory genes, repair genes, and stress signaling genes are all potential cancer drug targets. DNA microarrays analyzing genome-wide gene expression patterns are widely used in drug target discovery and provide leads to potential clinical applications (24). Other omic technologies are not far behind. Although new technologies are providing a wealth of riches in potential new cancer drug targets, the challenge is to determine which targets are driving the cancer and which may be secondary to the tumorigenic process and, in fact, not targets. The need, therefore, is for a functional genomics approach before target and compound selection in drug discovery. An example of how novel targets may be functionally elucidated is the work of Fesik and colleagues who used a combination of DNA microarray with siRNA transient knock-down of *Rb1*, mimicking the control of cell cycle through *Rb1* dissociation from E2F, as a functional genomics approach to elucidate the role of the *Rb1* (25). Genome-wide siRNA screening to study the biologic function of individual genes and their role in signaling pathways is being used as a new approach for target discovery and biomarker identification (14). Thus, the challenge for the future is not the number of potential cancer targets that can be identified, but the quality of the targets. This can only be assessed through appropriate mechanistic validation and ultimately clinical testing.

Not all molecular targets identified are druggable. Many oncogenes and tumor-suppressor genes encode transcription factors and the deregulated expression, activation or inactivation, and mutation and translocations of genes for transcription factors play critical roles in tumorigenesis (26). In addition, oncogenic signaling pathways frequently regulate sets of transcription factors that control the expression of families of genes resulting in tumor formation, progression and metastasis. Transcription factors except for ligand-activated transcription factors such as the nuclear hormone receptor superfamily, present major challenges for drug discovery (27). However, although they themselves may not be readily druggable targets, the pathways regulating them may be. Thus, the transcription factors NF-κB, HIF1-α, and p53 have proved to be druggable cancer targets. It is apparent that target validation requires a detailed understanding of not only the targets themselves, but the pathways regulating them.

Small-Molecule Drug Discovery

The process of drug discovery involves the identification of therapeutic candidates, their synthesis, characterization, screening, and assay for therapeutic efficacy. Once a compound has established its value in these tests, it undergoes preclinical development prior to introduction into clinical trials (Figure 43-1). Small-molecule therapeutics, typically considered to be compounds of less than 500-D molecular weight (although some natural product derivatives may stretch this limit), remain the most popular group of new cancer drugs being introduced into the clinic. Techniques for finding a

lead small molecule to inhibit a molecular target can include high-throughput screening (HTS) of chemical libraries (26). There has been considerable criticism of the low productivity of traditional HTS with million-plus compound libraries (29) and there has been a move to the use of focused libraries covering defined chemical space, natural product libraries that provide chemical scaffolds for further chemical modification, and fragment-based screening. The latter involves the use of lower molecular weight (120–250 D) and, thus, less functionalized compounds, with correspondingly lower affinities for the target (30). Fragment hits typically possess high ligand efficiency and are suitable for optimization into clinical candidates with druglike properties. However, because of their low affinities, fragment-based hits need to be screened at higher concentration using sensitive biophysical detection techniques such as protein crystallography and nuclear magnetic resonance (NMR) as the primary screening techniques, rather than bioassays. Another new approach for early-stage, small-molecule lead discovery is *in silico* computer-based screening (virtual screening) of target protein structures using libraries of up to 50 million compounds (31,32).

Antibodies

Antibody therapies have become an important component in the management of cancer. Recombinant technology offers enormous opportunities to tailor antibodies to meet clinical requirements, including the reduction of immunogenicity and the development of smaller antibody fragments that can be incorporated into fusion proteins. Antibodies can block tumor growth factors or their receptors, activate immunologic attack on the tumor, or be used to deliver payloads such as radioisotopes, cytotoxic drugs or toxins, and nanoparticles (33). Pretargeting approaches include streptavidin/biotin systems and antibody-directed enzyme prodrug therapy (ADEPT). ADEPT uses an antibody-enzyme complex to deliver a prodrug-activating enzyme to tumors for selective prodrug conversion at the tumor site. New antibody targets, refined antibodies, antibody fusion proteins, the use of antibodies in combination therapies and as adjuvant therapy are additional new ways of incorporating them into cancer treatment.

Immunotoxins and Related Molecules

Immunotoxins are proteins that contain a toxin linked to an antibody (or growth factor) that binds specifically to target cells (34). For an immunotoxin to work, it must bind to and be internalized by the target cells, and the enzymatic fragment of the toxin must translocate to the cytosol. At present, only one agent that contains human interleukin-2 and truncated diphtheria toxin is FDA approved, for use in cutaneous T-cell lymphoma. Another, containing an anti-CD22 Fv and truncated *Pseudomonas* serotoxin, has induced complete remissions in a high proportion of cases of hairy-cell leukemia (35).

Vaccines

Active, specific immunotherapy (vaccination) against cancer has a disappointing history. Numerous studies have been conducted

addressing issues related to the generation of a therapeutic immune response against tumors and exploring a diverse array of antigens, immunologic adjuvants, and delivery systems for vaccinating patients against cancer. However, the results have been frustratingly unpredictable. Reasons for this lack of progress include inadequate understanding of the nature of the immune response, poor vaccine design, and a lack of objective measures to evaluate efficacy (36). Despite this, data indicate that vaccine therapy is safe, and no significant autoimmune reactions have been observed even on long-term follow-up. The design of clinical trials for vaccines has not yet been optimized, but meaningful clinical effects have been seen in B-cell malignancies; lung, prostate, and colorectal cancers; and melanoma. It is also clear that patients with limited disease or in the adjuvant settings have benefited most from this targeted-therapy approach (37). New genetic technologies have advanced our ability to identify candidate tumor antigens, and we understand more about the activation and regulation of immunity against cancer. There are now vaccine strategies to activate specific attack on tumor cells, and assays using surrogate markers of performance to correlate with clinical effects.

Current areas of investigation include the discovery and characterization of novel antigens expressed preferentially or exclusively on tumors to be used as targets for vaccination; the investigation of different vaccine-delivery modalities such as cellular-based vaccines, protein- and peptide-based vaccines, and vector-based vaccines; the characterization of biologic adjuvants to further improve the immunogenicity of a vaccine; and the investigation of multimodal therapies in which vaccines are combined with other therapeutic modalities such as radiation and chemotherapy (38). There are of course successes of vaccination therapy for prevention of cancer. Hepatocellular carcinoma (HCC), the most common neoplasm in China, is credited as the first malignancy that can be largely eliminated by a safe antiviral vaccine and other transmission control measures (39). In 2000, 580,000 individuals died worldwide from chronic infection-related cirrhosis and HCC. Of these an estimated 84% or more could have been saved by routine infant hepatitis B vaccination (40). A recombinant human-papillomavirus-like particle vaccine has recently been approved by the FDA for use in girls and young women to prevent diseases associated with infection by human papillomavirus (HPV) types 6, 11, 16, and 18, including genital warts, precancerous cervical, vaginal or vulvar lesions, and cervical cancer (41).

Gene Therapy

Approaches to gene therapy include the transfer of tumor-suppressor genes; suicide genes—enzyme/prodrug approach; inhibition of dominant oncogenes; immunomodulation; expression of molecules that affect angiogenesis, tumor invasion, or metastasis; chemosensitization and radiosensitization approaches; and chemo-protection of stem cells. The clinical application of gene therapy has required the development of effective and safe DNA-delivery vehicles or vectors. Many approaches to cancer gene therapy have been proposed, and several viral and nonviral vectors have been brought to the clinic. Commonly used vectors include retroviral vectors, adenoviral vectors, adeno-associated viral vectors, pox viruses, herpes simplex viruses, HIV-vectors, nonviral vectors, and targetable

vectors (42). The efficacy of nonreplicative, first-generation adenovirus (Ad)-based cancer gene therapy was low and data from clinical trials disappointing. To address this problem, conditionally replicating Ads have been constructed (43).

RNAi

Our improved understanding of the molecular pathways important for tumorigenesis has created opportunities for cancer therapy using RNAi technology to target the key molecules within these pathways. The silencing of critical gene products for tumorigenesis and molecules crucial for tumor–host interactions and tumor resistance to chemo- or radiotherapy by RNAi technology has generated significant antiproliferative and pro-apoptotic effects in cell culture systems and in preclinical animal models. Problems that have to be solved before the approach can be successfully translated into the clinical area are efficient *in vivo* delivery, incomplete suppression of target genes, nonspecific immune responses, and off-target effects (15).

Preclinical and Clinical Development of Targeted Drugs

Drug Delivery

The use of nanoparticles as drug delivery vehicles has the potential to change the way cancer drugs are delivered to tumors. The repackaging of paclitaxel in nanoparticle formulation has already shown increased access to endothelial and tumor cells and the potential for enhanced therapeutic efficacy, compared with the conventional version solubilized in cremophor (44). Lipid-based nanoparticles (liposomes) are already in use as drug-delivery systems that protect the drug from degradation, improve its pharmacokinetic properties, and deliver a high drug payload. Although several liposomal formulations of chemotherapeutic drugs have been approved for cancer therapy, improved drug delivery by these liposomes is accomplished mainly by passive means, for example, by enhanced permeability and retention. In various stages of development are antibody immunoliposomes with the goal of cell-specific targeted drug delivery (45). Intracellular delivery and effective cellular uptake represent major challenges to the widespread use of RNAi *in vivo* due to the high-molecular-weight and negative charge of siRNA duplexes. An example of the way this is being overcome is the incorporation of siRNA to the transcription factor EphA2 into neutral liposomes which, in animal models, provides a highly effective means of reducing tumor EphA2 expression and antitumor activity, either alone or with increased activity when combined with paclitaxel (46). In the future, the abnormal structure of the tumor vasculature may allow the design of nanoparticles that preferentially accumulate at the tumor site and, if loaded with drug, to deliver their cargo directly to the tumor (47). Intracellular targeted delivery of drugs is also being developed through the use of pH-sensitive liposomes, cell-penetrating proteins and peptides, and immunoliposomes targeting intracellular antigens. Also being developed are delivery mechanisms to intracellular targets, for example the nucleus for gene

therapy agents, and the mitochondrion for pro-apoptotic drugs and targeting the mitochondrial genome (48).

Toxicology

Drugs fail either in preclinical development or, much more costly, in clinical development, usually because of a lack of efficacy or because of toxicity. With molecularly targeted agents there are three types of toxicity that can be expected: on-target toxic effects, on-target but unexpected toxic effects, and off-target toxic effects. An example of a nontarget toxicity is the skin rash due to inhibition by small-molecule EGF receptor inhibitors or antibody of EGF receptors in the skin (49). It has been suggested that it may be used to predict patients who will benefit from EGFR inhibitor therapy (50), although this remains to be proven in prospective studies. Another example of on-target toxicity is increased blood glucose resulting from inhibition of insulin signaling by inhibitors of the phosphatidylinositol-3-kinase/Akt/mTOR signaling pathway (51). Unexpected toxicity can be due to an on-target effect that is not seen in preclinical animal studies, for example the cardiac toxicity of imatinib (Gleevec), a small-molecule inhibitor of the fusion protein Bcr-Abl, which is the causal agent in chronic myelogenous leukemia. It has been observed that a low but significant number of patients receiving imatinib developed severe congestive heart failure (52). Of interest, retrospectively it was found that imatinib-treated mice also developed left ventricular contractile dysfunction. Given that other receptor kinases inhibited by imatinib in addition to Bcr-Abl, including platelet derived growth factor receptor (PDGFR), stem cell ligand receptor (c-kit), and macrophage colony-stimulating factor receptor (c-fms; 53,54), inhibition of one or more of these receptor kinases could be responsible for the cardiac toxicity seen. Off-target toxicities in clinical trials will always be difficult to predict and will depend on the efficacy of preclinical animal models for predicting toxicity and knowledge of the noncancer-related targets of the drug. Investigation of genetic and nongenetic differences in individual patients is important to identify the variables most important for optimal dosing (55). For example, genotyping *UGT1A1* to reduce the incidence of severe toxicity of irinotecan since *CYP3A4* and *UGT1A1* activity appear to be predictive of irinotecan toxicity rather than efficacy.

Toxicogenomics

The combination of the technologies of genomics and bioinformatics to identify and characterize mechanisms of action of toxic compounds is termed “toxicogenomics” (56). Toxicity profiling with DNA microarrays or by proteomics can be used to provide improved descriptors of toxicity, toxicant classification, and measures of exposure (57). Although toxicogenomics is in its infancy for cancer therapy, a shared goal with transcript and proteomic profiling of therapeutic agents is the development of biomarkers and signatures of chemical toxicity.

Pharmacokinetics

One of the reasons for drug failures during development is sub-optimal pharmacokinetics. Efficacy failures can occur through

too low a concentration of drug reaching the target for an inadequate amount of time. Safety failures can occur because of the wrong concentration reaching the wrong target for too long a time period. A full understanding of the kinetics of a drug, thus, retains a central place in targeted drug development, and it is important to have pharmacokinetic studies built in early in the drug development process. It is important to know, for example, that concentrations of drug in the blood can be maintained at high enough levels and for a sufficient time to ensure inhibition of the molecular target. Early evidence of pharmacokinetic properties can also guide dose, scheduling, route of administration, and how to best to combine an agent with other drugs. Pharmacokinetic studies are often combined with pharmacodynamic studies that directly measure molecular target inhibition in the tumor and in normal tissues through assays on biopsies or by molecular imaging studies. Although animal pharmacokinetic studies are important during drug development there is no substitute for pharmacokinetic studies in patients. Animal and in vitro pharmacokinetics studies used to predict human pharmacokinetics have been found to be incorrect in approximately one in three occasions. Bioavailability is often poorly predicted, as are drug phase 2 conjugation pathways (7). Thus, assessment of human pharmacokinetics is an essential component for cancer drug discovery.

Pharmacogenomics

The same doses of drug cause considerable heterogeneity in efficacy and toxicity across human populations, and genetic factors are thought to represent important determinants of both drug efficacy and toxicity. Pharmacogenetics focuses on the prediction of the response of tumor and normal tissue to standard therapy by genetic profiling and, thereby, to select the most appropriate agent at optimal doses for each individual patient. This can include single-nucleotide polymorphisms (SNPs) in genes, whose gene products act upstream of the actual drug target sites such as drug transporters and drug metabolizing phase I and II enzymes, or downstream of them, including apoptosis-regulating genes and chemokines (58). The discovery of clinically predictive genotypes (e.g., UGT1A1, TYMS TSER), haplotypes (e.g., VKORC1 haplotype A) and somatic mutations (e.g., epidermal growth factor receptor), along with the introduction of FDA-approved pharmacogenetic tests (UGT1A1) and the initiation of a genotype-guided clinical trial for cancer therapy (TYMS TSER in rectal cancer) have provided the first steps toward the integration of pharmacogenomics into clinical practice (59).

Imaging as a Guide to Therapy

Computed tomography (CT) and magnetic resonance (MR) imaging have facilitated drug development by providing quantifiable and objective evidence of response to cancer therapy. More recently metabolic imaging using [^{18}F]-fluorodeoxyglucose-positron emission tomography (PET) has allowed for earlier detection of response. Newer molecular imaging techniques use noninvasive modalities, radioligands, and contrast agents measure functional and molecular events in tumors by targeting

genes and proteins linked directly to cancer (Table 43-3; 60–62). It is also possible to image pharmacokinetic and pharmacodynamic changes produced by therapeutic agents in the tumor CT, MR, and PET imaging along with ultrasound and optical imaging such as bioluminescence, fluorescence, near-infrared imaging, and multispectral imaging have become increasingly used in preclinical studies in animals to document the effects of genetic alterations on cancer progression or metastases, for the detection of minimal residual disease, and response to various therapeutics including radiation, chemotherapy, or biologic agents. Molecular imaging offers potential to deliver a variety of probes that can image noninvasively drug targets, drug distribution, cancer gene expression, cell surface receptor or oncoprotein levels, and biomarker predictors of prognosis, therapeutic response, or failure (63).

Clinical Trials

The information derived from clinical trials is critical for new drug development and the discovery of the next generation of cancer drugs. New advances in clinical trials of cancer drugs are microdosing and the rational combination of molecularly targeted drugs.

Microdosing

A new type of clinical trial has recently been introduced for exploratory IND studies, sometimes called “phase 0” clinical trial or microdosing, as part of the FDA’s Critical Path Initiative to modernize the clinical trials process. It allows researchers to quickly establish

Table 43-3 Examples of Imaging Techniques

Magnetic resonance Dynamic contrast enhanced (DCE) MRI Iron oxide nanoparticle MRI DCE computed tomography MR spectroscopy	Angiogenesis, vascular permeability Malignant lymphadenopathy Tissue perfusion, vascular permeability Metabolism
Ultrasound (US) US contrast agents (microbubbles)	Intravascular volume
Positron emission tomography (PET) H_2^{15}O -PET ^{18}F FDG-PET ^{125}I UdR-PET ^{18}F FLT-PET	Blood flow Metabolism DNA synthesis DNA synthesis
Single photon emission tomography (SPECT) $^{99\text{m}}\text{Tc}$ -annexin V $^{99\text{m}}\text{Tc}$ -endostatin $^{99\text{m}}\text{Tc}$ -celecoxib $^{99\text{m}}\text{Tc}$ -metronidazole	Apoptosis Angiogenesis COX-2 expression Hypoxia
Labeled antibody Biotinylated/avidin-Gd-DTPA $^{99\text{m}}\text{Tc}$ -EGF receptor antibody	HER2 receptor EGF receptor

Source: Atri M. New technologies and directed agents for applications of cancer imaging. *J Clin Oncol* 2006;24:3299–3308; Czernin J, Weber WA, Herschman HR. Molecular imaging in the development of cancer therapeutics. *Ann Rev Med* 2006;57:99–118; and Yang DJ, Kim EE, Inoue T. Targeted molecular imaging in oncology. *Ann Nucl Med* 2006;20:1–11, with permission.

whether an agent of interest works as desired within humans and, thus, could potentially offer a clinical benefit (64). The strategy requires fewer preclinical animal studies than a typical phase 1 clinical trial and allows researchers to make smaller batches of an experimental drug under relaxed guidelines. To offset safety concerns, the human tests are limited to a few subjects, a short time frame of no more than 7 days, and a reduced dose to ensure no adverse toxic effects. Microdosing, at 100th of the pharmacologic dose determined from animal models and in vitro systems, whichever is less, has been available to European researchers for several years. It is unlikely that target inhibition will occur at these doses but uptake at the target level may be assessed and important human pharmacokinetic information may be obtained. The ability to obtain basic pharmacokinetic parameters such as clearance, volume of distribution, and $t_{1/2}$ from microdosing is dependent on the availability of ultra-sensitive analytical methods that can measure drug and metabolite concentrations in the low picogram to femtogram range. A concern is that a microdose may not predict the behavior of clinical doses, although there is some evidence that for many drugs linearity or near-linearity is approached during microdosing (65).

Drug Combinations

A rational way to use molecularly targeted cancer drugs is combinations that attack different points in the same or parallel signaling pathways. A frustration for many clinical investigators wishing to move toward the goal of rational therapy is the reluctance of pharmaceutical companies to test their experimental agent in the clinic together with a competitor's experimental agent. A major step to overcoming this problem has been taken by the Cancer Therapy Evaluation Program (CTEP) of the National Cancer Institute as part of the Critical Molecular Pathways initiative, which focuses on combining investigational agents in the EGFR and phosphatidylinositol-3-kinase signaling pathways. CTEP guidelines provide intellectual property protection to companies that test drugs in combination and CTEP provides the framework for conducting these studies (66). A number CTEP-sponsored drug combination studies are under way. Since many of the newer cancer drugs are cytostatic and do not cause tumor shrinkage, it is clear that they will need to be given in combination with cytotoxic drugs if tumor shrinkage is the goal.

Academia and Innovative Science

Drug targets and the rationale for new drug therapies are often identified by academic scientists. Actual drug discovery and development has traditionally been conducted by pharmaceutical companies. The reasons usually given are the nonhypothesis-driven nature of the work, the requirement for personnel with multidisciplinary expertise, and the high cost (7). Although discovery and study of the biology of potential new drug targets will continue to be a realm for academic researchers, the landscape for drug discovery and development is rapidly changing. With molecularly targeted therapeutics, the discovery process is

now very much hypothesis driven. At the same time understanding the intricacies of the targets being studied at the molecular and systems biology level, traditionally one of the strengths of academic research, is very much part of modern drug discovery. So why not combine the two processes? Even relatively small academic institutions have investigators or collaborators from a range of disciplines, often a greater variety than found in large pharmaceutical companies. Finally, the costs of drug discovery are comparable to those of many academic or government-sponsored core facilities. One of the major strengths of the pharmaceutical industry has been the large numbers of chemists that can be brought to focus on a single drug discovery project. With the new technologies of computer-based in silico drug screening and rational drug design, as well as high-throughput screening for lead molecules available as core facilities for academic researchers through the Molecular Libraries Screening Center Network of the National Institutes of Health Roadmap for Medical Research initiative (67), academic investigators can realistically expect to be able to discover lead molecules for their molecular targets. Chemistry is still needed to make the compounds, but innovative approaches such as outsourcing to specialist service companies, as the pharmaceutical industry is itself doing, is also available to academia. A variety of computational algorithms have been developed to aid the drug development process and predict drug properties, from physicochemical properties, ADME (absorption, distribution, metabolism, and elimination), and blood-brain barrier penetration (68,69). The rapid advances in nanobiotechnology drug delivery, pharmacokinetics and pharmacogenomics, toxicokinetics, and toxicogenomics derive in large part from academic groups. The challenge is to effectively harness and organize the staggering range of multidisciplinary expertise found in academia for the drug discovery and development process, but it can be done. It is not beyond the capabilities of academia to conduct the preclinical safety and product stability studies required by regulatory agencies such as the FDA. There is also assistance to be found for toxicology and clinical drug product manufacture from federal programs such as the NCI Rapid Access to Intervention Development (RAID) program and the NIH Roadmap for Medical Research Initiative. The majority of the cost of bringing a new drug to registration is clinical trial costs (7). Realistic goals for academic drug discovery include discovery and development of novel agents against novel molecular drug targets and partnering with industry for clinical development. Given the current reluctance of the pharmaceutical industry to license in new agents until phase 2 clinical trials in most instances this may have to include phase 1 clinical trials sponsored by the academic institution, which would be an investment in advancing their intellectual property.

Conclusion

This overview of new drug discovery and development provides an introduction to detailed presentations in the following chapters. The past few years have witnessed huge changes in the approach

to creating new anticancer agents, in part due to sophisticated new technologies and computer tools, and in part due to new ways of identifying abnormal genes and molecular pathways in cancer cells that can be validated as potentially fruitful targets. Today, the menu of potential therapeutic agents that can target genes and molecules includes DNA, RNA, peptides, proteins, and antibodies, in addition to small organic molecules that have formed the backbone of cancer chemotherapy for many decades.

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Screening Strategies for Targeted Therapeutics

The concept that chemicals might be useful in the treatment of cancer arose in part from the speculations of, for example, Nobel Prize winner Paul Ehrlich, that “magic bullets” might be defined that would have selective activity in or affinity for tumor as opposed to host compartments. This notion arose from the observation that certain chemical dyes could be defined that reacted with distinct organs and tissues or subcellular compartments. Initial efforts to define potential antineoplastic agents used “empirical” screening strategies. That is, compounds of interest were initially detected by their ability to inhibit tumor cell growth in vitro or in animals (in vivo screens) bearing test tumors (e.g., mice with the syngeneic L1210 leukemia). Historically, these screens were conducted at universities or research institutes (Harvard, Memorial Sloan Kettering) during the 1930s through 1950s (1). The initial clinical successes with nitrogen mustard derivatives in the treatment of certain lymphomas and leukemias (2), and in folate antagonists in the studies of Farber et al. (3) in pediatric patients with acute lymphoblastic leukemia encouraged the view that cancer could be effectively addressed by chemotherapy and raised the possibility that the interest of the corporate and academic spheres would not be well served by the existing screening outlets. In 1955, the U.S. Congress directed the National Cancer Institute (NCI) to set up a screening and evaluation program that would allow the rapid assessment in clinical trials of promising chemicals for the treatment of cancer (4). As a result, the cancer chemotherapy National Service Center was established, followed shortly thereafter by the predecessor of today’s Cooperative Group Clinical Trials networks.

The detailed history of the NCI drug screening and evaluation systems has been presented elsewhere (5) and will not be repeated here. Although designed without any reference to the biologic bases of cancer, empirical drug screening systems have yielded clinically useful drugs, including such entities as the classical alkylating agents, anthracyclines, taxanes, camptothecins, and so forth and have thus been responsible for empirically based regimens that have contributed to curative treatments for lymphomas, leukemias, and germ cell neoplasms, as well as meaningful activity in epithelial neoplasms in combination with surgery or radiation or when given in the adjuvant setting.

One of the most important predictors for ultimate clinical value of agents selected by such empirical screening programs is the behavior of the drug candidate in animal models of cancer, usually mice bearing syngeneic or xenografted human tumors. In

one review of the NCI experience, agents with evidence of activity in more than 33% of animal models tested had a 50% likelihood of activity in more than one phase 2 trial (6). Yet there was poor correspondence of activity in a particular animal model histology compared with activity in the same human cancer type in the clinic. This finding was in general recapitulated by an independent data set from NCI-Canada (7). However, it is profoundly unsatisfying that these historic in vivo screening approaches in no way incorporated a biologic understanding of the bases for a tumor’s growth and progression, albeit the early screening efforts occurred at a time when such knowledge was scant. Approaches to cancer drug discovery are seeking to modify this process to incorporate biology at an early point in the discovery process, and then qualify a compound for further development by assaying a continuing capacity of evolved versions of the drug to “hit” its molecular target even as its structure is being optimized for useful pharmacological and pharmaceutical features. Figure 44-1 contrasts the empirical and targeted drug development approaches preclinically and in early clinical development.

Molecular Targets for Cancer Treatment: Implications for Drug Discovery

The molecular revolution in our understanding of the origins of cancer and its progression has brought recognition that successful cancers possess mutations leading to activation of autonomous cellular proliferation: loss of cell death promoting mechanisms, loss of counter-growth mechanisms, the acquisition of limitless replication potential, and the ability to invade, metastasize, and alter host stroma to promote the progress of the tumor (8). Accordingly, each of these processes could potentially embody the basis for useful cancer treatments.

How to recognize potentially useful targets for cancer treatment is the subject of much debate. One point of view is that in order to be successful, a target must be critically important to the “molecular economy of a tumor” so that removal of the target would result in a state incompatible with tumor cell survival. In essence, the tumor is “addicted” to the presence of the target (9). One way to recognize such targets of import is hypothesized to be the presence of mutations in a particular pathway. Indeed,

	EMPIRICAL	TARGETED		EMPIRICAL	TARGETED
Basis for screen:	Effect on growth in vitro/vivo	Biochemical or "Model" organisms	Agent selection:	Murine tumors Xenograft models	Credentialed targets molecular models
Priority:	Number of models affected	Growth inhibition tied to target occupancy	Priority:	Log cell kill	Growth inhibition
Optimize:	Chance improve PK/activity	Structure of target $\pm$ effect on target	Trial design:	Empirical	Target/hypothesis
Endpoint eval:	Tumor size	Complex assays	Dose endpoint:	Toxicity	Molecular effect When to measure?
Toxicology:	Tie to PK	Tie to target effect and PK	Endpoint eval:	H&P, clin. lab	Complex assays
A			Eligibility:	Any solid tumor	Presence of target
			B		

FIGURE 44-1 Comparison of empirical and molecularly targeted cancer drug screening strategies. Whether at the preclinical (**A**) or clinical development (**B**) stages of development, the targeted approach seeks to incorporate knowledge of the candidate drug's action on its target, or the presence of the target, to inform the development strategy.

emerging clinical evidence supports this point of view. This is exemplified by the success of imatinib in the treatment of chronic myelogenous leukemia (with activation of abl kinase signaling through translocation and production of a chimeric fusion protein; 10) and the recognition that non-small cell lung cancer patients responding well to epidermal growth factor receptor antagonists frequently have mutated, activated epidermal growth factor receptor (EGFR) signaling (11). By this thinking, the best cancer targets effectively behave in a fashion analogous to "synthetic lethal" mutations in yeast, where the mutation is not revealed except in the context of another mutation or stress. By this way of reasoning, drug action renders nonfunctional a target which causes lethality only in the context of the drug (12).

Strategies for drug discovery that would depart from knowledge of such important targets might not actually consider demonstration of antitumor activity in animals until a relatively late stage in the screening process. In this approach, much energy is spent defining lead structures that are able to bind to and influence the function of oncologically relevant targets in vitro or model systems. Compounds can then be optimized with constant reference to the ability of the compound to affect its putative target.

Process of Drug Screening and Discovery

The essence therefore of a cancer-relevant screening program is how to recognize new molecular entities that will have value as anticancer drugs. As stated previously, screens may depart from *empiric* assays of cellular proliferation in vitro or in vivo, or from *molecularly targeted* assays against particular, purified molecules, or cells modified to possess the relevant drug target (Figure 44-2). Conventionally, thousands to millions of candidate molecules are evaluated in such screens. "Hits" are molecules that score positive by some criteria that will produce a manageable number of follow-up candidates. Hits are considered and may be discarded owing to rarity in nature of the originating source (if not a synthetic chemical), impracticality of structure for scale-up synthesis or production, or chemical incompatibility with administration to the host. "Leads" are molecules that survive secondary screens designed to remove compounds that although active in the initial screen, nonetheless have undesirable properties such as activity against other

targets, poor selectivity, low potency, and so forth. "Lead optimization" refers to successive chemical modification to impart druglike properties to the evolving lead. These include potency, ability to be manufactured, capacity to be distributed into a tumor, potential to be absorbed and eliminated without toxicity, ability to be placed in a stable formulation compatible with appropriate solubility, or

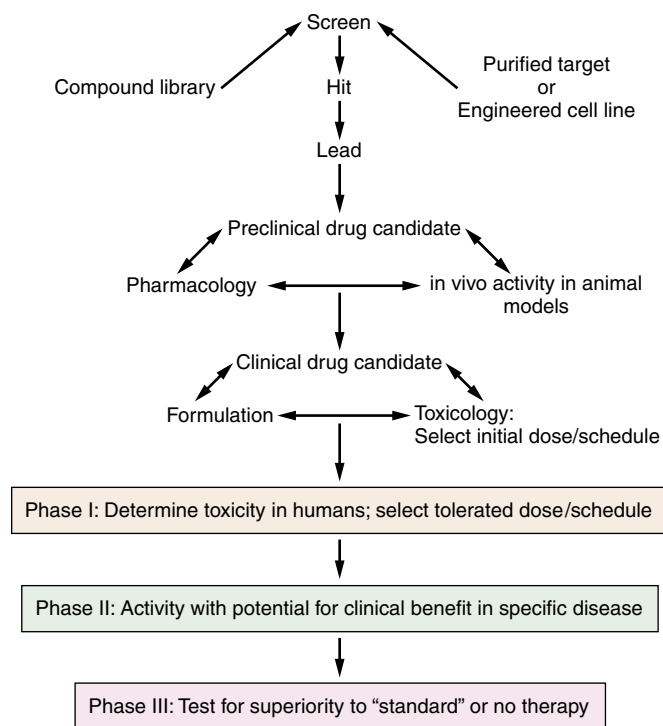


FIGURE 44-2 General process of targeted drug discovery and development. Knowledge of a target allows creation of purified molecules or transfected cell lines, which are combined with collections of candidate compounds to undertake a screen from which "hit" compounds are identified. After considerations such as cost of acquisition, likely tractability in physiologic fluids, deconvolution of mechanism if detected by screening in cells, and chemical class, leads may be defined that are conscientiously optimized to achieve desired pharmacology in models that reflect target action. This allows the definition of a limited number of clinical development candidates, which are studied to produce formulations with desired properties (e.g., oral bioavailability), which are then qualified in safety testing toxicology studies to define a starting dose for phase 1 testing. Guidance by effects on the drug molecule's target can contribute to an understanding of the relation between plasma levels and likely efficacious action on the target in model organisms. Completion of phase 1 testing allows progress to later stage of development in patient populations preselected to have target expression of importance of function in their tumors.

capacity for administration. This process reflects a continuing cycle where chemists modify the lead, preserving the “chemotype” or “pharmacophore” that retains biologic and/or biochemical activity; biologists study the drug’s activity; pharmacologists its distribution and elimination properties; and ultimately safety testing toxicology assures likely safety of the drug in initial clinical studies conducted under a U.S. Food and Drug Administration (FDA)–reviewed Investigational new drug (IND) application. This process is intrinsically expected to discard greater than 90% to 95% of compounds considered as “positive” in early screens. At best, one in three to at worst one in 20 early clinical candidates lead to approved drugs (Figure 44-2).

Empiric Screening Approaches

Purely empiric screens present pure compounds or mixtures of compounds to cells proliferating in tissue culture or animal tumor model systems. The success of screening is thought to be dependent on the quality of the sample libraries screened and the screening models themselves. Natural products (defined as extracts from plant or bacterial origin) figure prominently as sources of approved drugs both cancer and other indications, because these sources contain a rich diversity of bio-active chemical compounds. However, the process of bioassay-directed natural product isolation (where pure compounds are ultimately identified from such sources) and characterization is time-consuming and labor-intensive. Additionally, the purified active constituents may not be amenable to synthesis or production from natural sources.

NCI has developed and operated a classic anticancer drug screen based on the use of panels of tumor cell lines representing a variety of tumor types, the so-called NCI60. In this model, each compound is tested in concentration-response fashion in each cell line, generating a profile of response across the cell line panels. This response profile serves as a “fingerprint,” which has been shown to reflect mechanisms of growth inhibition or cell killing. Informatic tools to decode these profiles and relate activity to archived data on potential molecular targets presented by the NCI60 tumor cell lines (COMPARE analysis and self-organizing maps) are publicly available at <http://dtp.nci.nih.gov>. The rationale, history, development, and operational results of this screen have been reviewed (13,14).

Compounds identified by empiric screening are then optimized by altering structures to improve pharmacologic and pharmaceutical properties while leaving the compounds’ ability to cause antiproliferative effects in animal models intact. Although deconvolution of mechanism of action may aid this process, in the past drugs identified by this route on occasion have proceeded to clinical evaluation with little understanding of their mechanisms of action, a circumstance that would be very unusual in the current age.

Molecularly Targeted Screening Approaches

As described previously, with the recognition that cancer depends on the operation of mutated growth regulatory genes,

attention in cancer drug screening has shifted to seeking recognition of initial lead structures directed against important targets. Molecularly targeted screens may be cell free, with purified biochemical reagents, or cell based, where the targets are presented operating in the context of usually an engineered cancer cell. It is even possible to create engineered organisms, such as yeast (15).

Whether cell free or cell based, molecularly targeted screening strategies have over the past two decades shifted in the commercial sector to libraries of synthetic compounds. Indeed, the use of “combinatorial chemistry” has been cited as a means of generation of synthetic libraries representing immense chemical diversity. Development of high-throughput screening (HTS) technology using robotic liquid handling systems and automated plate readers has made it feasible to process larger and larger libraries in relatively short time frames (16–18). Commercial computer software now available can support both the screening process and chemo-informatic analysis of screening results. The general process of high-throughput screening is illustrated in Figure 44-3. Drug design efforts have benefited from development of advanced computer tools. Quantitative structure-activity analysis (QSAR) can speed optimization of drug leads obtained from screening or factor as a tool in analog development. Molecular modeling software can exploit structural information regarding target molecules to optimize drug leads or perform *in silico* screening of virtual chemical libraries.

Cell-Free Screening

Cell-free, biochemical, or biophysical screens can provide very simple and precise screening platforms consistent with a high degree of assay miniaturization and automation. Indeed such screens can be reduced to assay volumes as small as two microliters using commercially available equipment. They have a theoretical advantage over cell-based screens in that targets are presented in isolation from cellular components (membranes, potentially redundant targets, competing reactions, etc.) and thus produce hits that clearly affect the target of interest. This can also be something of a disadvantage, since such screens can yield active, targeted compounds that cannot cross cell membranes and thus require chemical lead optimization to support further development. Selectivity of active compounds can be defined by screening against related targets (counter screening) and prioritizing leads that have a desired degree of specificity.

Two types of initial cell-free screening approaches might be defined. In the first, molecular recognition of a macromolecule, without initial reference to affecting function, is the goal. By defining aspects of a pharmacophore that can interact with a novel target, a basis for then combining the elements of the pharmacophore to yield higher affinity binding can then be approached. Examples of this might include nuclear magnetic resonance (NMR)–based screening approaches that have yielded potent inhibitors of bcl2-related BH3 domain interactions (19). Alternatively, chemical interaction approaches define substructures with affinity for some feature or active site (e.g., Tether technology; 20). This results in

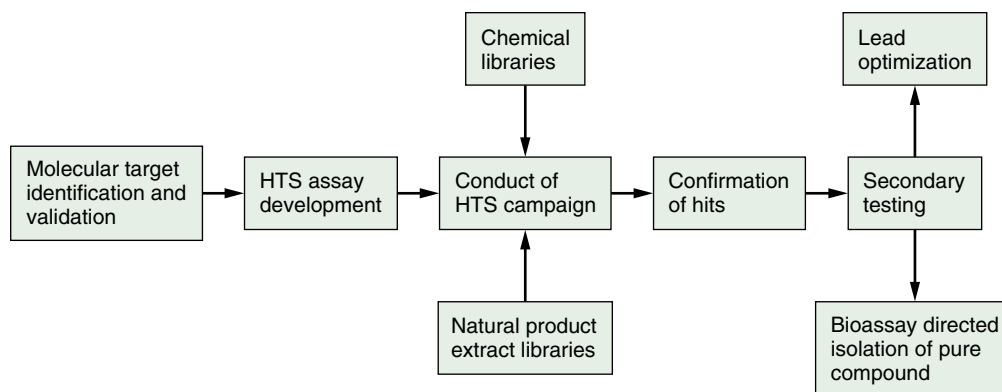


FIGURE 44-3 GENERAL PROCESS FOR HIGH-THROUGHPUT DRUG SCREENING. Once a molecular target has been identified and validated, an assay consistent with high-throughput screening (HTS) is developed and characterized. This typically entails adaptation of a research method to a plate-based automated assay. Production and quality control of sufficient amounts of reagents, especially target proteins, are frequently rate-limiting in this process. Reproducibility must be demonstrated and quality control established. The assay can then be coupled to chemical or crude natural product libraries for conduct of screening campaigns. This is typically done using a single concentration of test material. Since all assays are associated with some rate of false-positive “hits,” these must be confirmed by retesting in the primary assay or in concentration-response assays. Confirmed hits can then be subjected to secondary testing to generate additional data to allow prioritization of leads for chemical optimization (in the case of synthetic libraries) or bioassay-directed isolation and characterization (in the case of crude extract libraries). (From Shoemaker RH, Scudiero DA, Melillo G, et al. *Application of high-throughput, molecular-targeted screening to anticancer drug discovery*. *Curr Top Med Chem* 2002;2:229, with permission.)

the ability to create higher affinity binding molecules that then recognize the target by noncovalent interactions.

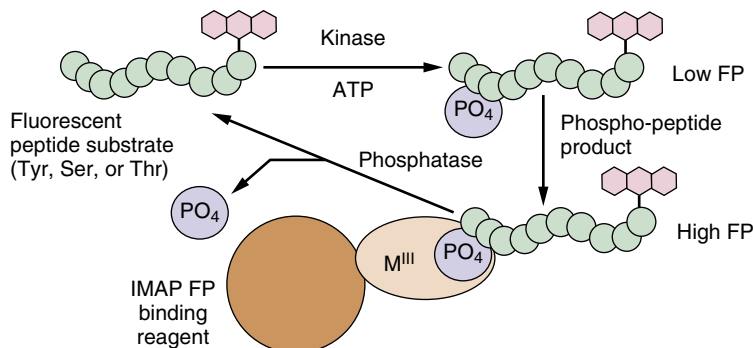
Alternatively, initial recognition of a drug candidate may proceed by its ability to alter the function of a target. The screening assay is therefore based on the biochemical action, usually enzymatic, of the target. The pharmaceutical industry has long favored biochemical, especially enzymatic, screens for nononcology applications. Indeed, the science of drug screening, and particularly high-throughput screening, has evolved to the point where specialized organizations have been established to support and advance the process (e.g., the Society for Biomolecular Screening, www.sbsonline.org). Some examples for specific types of cancer-targeted assays include the cases described in the following paragraphs.

The prominence of kinases as key molecules in cell signaling has vaulted them to the fore as drug development targets for a variety of indications including cancer. More than 500 kinases have been identified in the human “kinome” and interest has been expressed in finding inhibitors for each one. The example of imatinib cited previously provides an example of a truly validated drug discovery target, in that clinical benefit has been associated with modulation of the kinase activity. In the laboratory, the activity of kinases has been classically studied in assays using radioactive phosphorus (^{32}P or more recently ^{33}P) with detection by autora-

diography. Although such assays can be very sensitive and can be the basis for elegant kinetic studies, they are not useful for high-throughput screening. This situation has spawned development of numerous alternative assays using some novel technologies. In one such technology, a peptide substrate is labeled with a fluorescent tag and, when phosphorylated, can be captured using microscopic beads (specially treated beads from Molecular Devices, Sunnyvale, CA). The capture results in a change in fluorescence polarization signal of the fluorescent tag which can be the basis for a screening assay. Thus, a cell-free assay can be constructed which includes only recombinant kinase, peptide substrate, beads, buffer (containing ATP), and screening sample. The principle of the assay is illustrated in Figure 44-4. An example of a protocol for an assay using commercially available CHK2 kinase is shown in Figure 44-5. A very similar assay using RSK2 kinase has supported characterization of inhibitors identified from molecular modeling (21).

Numerous other approaches to nonradioactive assays for high-throughput screening have been developed. Fluorescent assays, although frequently very economical, sensitive, and convenient, are not without problems. Screening sample fluorescence can significantly interfere with assays, particularly when screening campaigns are conducted at concentrations above $1\ \mu\text{M}$. The use of polarization, as illustrated previously, mitigates this interference

FIGURE 44-4 Illustration of the bead-based assay principle. Following phosphorylation of a fluorescent peptide substrate, specially treated beads are used to capture the peptide, resulting in an increase in the fluorescence polarization signal of the complex. The same principle can also be used to assay phosphatase activity which yields a decreased signal. (MDS Analytical Technologies).



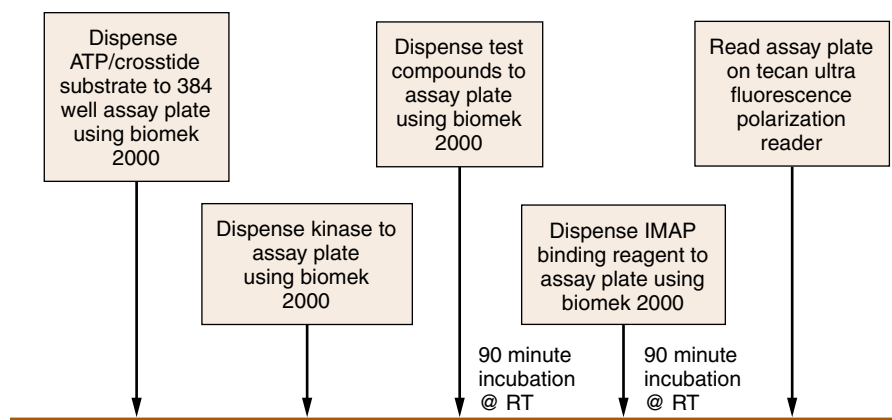


FIGURE 44-5 Schematic diagram for a CHK2 kinase assay using IMAP beads and fluorescence polarization detection. Reagents are dispensed with an automated liquid handling device into 384-well assay plates, incubated, and read on a plate reader.

to some degree. Use of time-resolved fluorescence is even more effective in this regard.

Phosphatases provide opposing action to kinases in cell signaling pathways and thus may also be attractive as molecular targets. However, structural studies have suggested that kinases are more “druggable” targets (i.e., they present binding pockets and cavities amenable to inhibitor action). Nonetheless, certain phosphatases with relevance to cancer biology have been the subject of drug screening campaigns. A variety of nonradioactive assays for phosphatase activity have been developed. Perhaps the simplest relies on quantitation of the complex formed between Malachite Green, molybdate, and free orthophosphate in solution. A colorimetric assay for this green complex can be used for high-throughput screening (22). Another approach is a fluorescence method using the artificial substrate 3-O-methylfluorescein phosphate (OMFP). A screen using this methodology has identified novel inhibitors of Cdc25B phosphatase (23). Inhibitors of Wip1 phosphatase were found in a screen conducted using the IQ Phosphatase Assay Kit (also fluorescence-based) produced by Pierce (24).

Although generally viewed as challenging targets for drug discovery, protein–protein interactions can be effectively addressed. Key proteins involved in the apoptotic process such as SMAC/DIABLO and XIAP have been identified as potentially important targets (25,26), HTS assays have been developed (27), and selective inhibitors identified (28–30). The assay used by the latter investigators was indirect, relying on caspase activity as detected by cleavage of a fluorogenic peptide substrate and was notable in that the combinatorial libraries screened amounted to approximately 1 million compounds. Schimmer et al. (31) reviewed therapeutics with potential in this target area, including a discussion of information supporting target validation. As described by Vassilev (32), a series of compounds termed “Nutlins” were “the first potent and selective small-molecule inhibitors of the p53-MDM2 interaction [and] have been identified recently by high-throughput screening followed by structure-based optimization.” The availability of Nutlins for detailed investigations of the p53-MDM2 pathway has supported identification of additional targets that may allow rational selection of patients for clinical study (33,34).

Proteases have been implicated in various aspects of cancer biology. Action of extracellular proteases has been considered in

relation to tumor metastasis and indeed, clinical trials of metalloprotease inhibitors have been conducted. High-throughput screens for protease inhibitors can be readily constructed using fluorescence resonance energy transfer (FRET) technology. For example a peptide substrate can be labeled with a fluorescent tag and a quencher molecule, such that, following cleavage by a protease, a fluorescent signal is detectable. This was done in the case of the anthrax lethal factor that acts as a protease to affect signaling in the MEK pathway to identify small-molecule inhibitors (35). As mentioned previously, inhibition of caspase activity has been assessed using cleavage of a fluorogenic substrate (29).

Cell-Based Screening

Cell-based screening assays can present molecular targets in the context of membrane barriers, cellular metabolism, subcellular localization, competing reactions, and extracellular fluids (serum remains a constituent of most culture medium used in screening). Thus, active compounds identified in cell-based screens will already have demonstrated the ability to penetrate cells or affect receptors. Cell-based assays also tend to tolerate crude natural product extracts that would interfere with cell-free assays. For example, many plant extracts contain tannins that can cross-link target proteins and generate an unacceptable rate of false-positives. A disadvantage of cell-based assays is that the detailed mechanism of action of active compounds must be defined in subsequent testing. Thus, although a cell-based screen may inform on modulators of a particular pathway, there may be many points in the pathway where an antagonist or agonist could act.

Engineered reporter systems are among the most popular cell-based methodologies for modern HTS. These have evolved from molecular biology tools using chloramphenicol acetyl transferase (CAT) and β -galactosidase reporters to the modern luciferase reporters that are currently in wide use. It is possible to design or purchase these in a variety of formats using both single and dual luciferase reporters. Bi-cistronic reporters using firefly and renilla luciferase genes can be used to generate normalized data. For HTS, firefly luciferase reporters provide a relatively simple assay format in which transformed cells are exposed to screening samples and then lysed and provided with luciferase substrate in a single step.

Automated plate readers are available to read such assays at densities of up to 1,536-well plates. Green and red fluorescent proteins have also been used as transcriptional reporters. Compared with luciferase systems, these tend to have lower signal-to-noise ratios and are subject to interference by sample fluorescence.

A transcriptional reporter system using firefly luciferase has been used to identify inhibitors of hypoxic cell signaling (36). A glioblastoma cell line (U-251) was transformed with an expression vector containing multiple copies of the hypoxia inducibility factor-1 α (HIF-1 α) response element (HRE) and a coding sequence for luciferase. When this cell is placed under conditions of low oxygen, HIF-1 α is stabilized, dimerizes with HIF-1 β , translocates to the nucleus and induces expression of luciferase. This can be conveniently detected on a plate reader as emission of photons following re-oxygenation of the cells, lysis, and exposure to luciferin substrate. Since HIF-1 α is a key regulator of hypoxic cell signaling and controls numerous downstream genes related to glucose uptake and metabolism, angiogenesis, and metastasis it is anticipated that leads from this screen could affect these processes in a potentially therapeutic way. Indeed, their secondary testing includes inhibition of VEGF expression and secretion to qualify compounds for further study. Remarkably, DNA topoisomerase I inhibitors were found to be relatively selective inhibitors of hypoxic cell signaling and a clinical trial of topotecan as a modulator of HIF-1 α is currently in progress.

The advent of gene-knockout technology has made it possible to create isogenic pairs of tumor cell lines that differ at only a single-gene locus (allele). Thus, genes thought to play a critical role in tumor biology can be targeted for creation of isogenic cell line pairs that can be used for drug screening. In an elegant implementation of this strategy, a colon cancer cell line was knocked out for a mutant Ras allele and transfected with an expression vector coding for yellow fluorescent protein. The wild-type cells were transfected with blue fluorescent protein such that the cell populations could be pooled, exposed to screening compound libraries, and read on a fluorescence plate reader at two wavelengths to identify compounds selectively affecting one member of the isogenic pair (37). In a screen of $\approx 30,000$ compounds from a commercial source and also some from the NCI repository, several compounds showing selective inhibition of growth of the mutant Ras-containing cell line were identified, including two chemotypes not previously known to affect the Ras signaling pathway.

The potential for construction of "phenotypic" screens seems limited only by the imagination of the investigator. Changes in a cellular phenotype upon challenge with a chemical library can provide leads for modulators or direct acting compounds. In another example, Smukste et al. (38) identified novel potentiators of doxorubicin action in E6-expressing colon cancer cells. A high-throughput screen of $\approx 26,000$ compounds identified several active chemotypes. As a cell-based screen, the activity could be mediated via a number of possibilities. These investigators prioritized leads termed "indoxins" for detailed investigation. Affinity tagged versions of these molecules were used to bind and cross-link candidate target molecules, which were subsequently captured and characterized as part of the detailed mechanistic follow-up. Two proteins were implicated as mediating the up-regulation of topoisomerase II, which was observed following treatment with indoxins.

The term "high-content" screening has been used to describe screens where detailed information regarding a target is collected, usually via an automated scanning microscope. A variety of instruments are now commercially available which include software for capturing and analyzing images. Although the throughput of such systems is relatively limited, they do provide a unique means of addressing targets such as nuclear translocation of transcription factors or other proteins. For example, Wolff et al. (39) used redistribution of an AKT-Green fluorescent protein (GFP) fusion protein within cells as a means of identifying modulators of PI3 kinase (PI3K) activity. Interestingly, a novel inhibitor of the PI3K pathway was recently identified by Japanese investigators using a 39 tumor cell line panel and informatics rooted in the COMPARE analysis that accompanies the NCI60 screen. This inhibitor, ZSTK474, is orally available and shows good activity in human tumor xenograft models. This antitumor activity was associated with inhibition of AKT phosphorylation in tumors as demonstrated by immunohistochemistry (40).

Screen Validation and Criteria for a Valuable Screen

The ultimate validation of a drug screen is the elaboration of product approved for clinical use. Contributions to this outcome require molecules with potency and selectivity for the target, but also require critical success with respect to features that are independent of the target's importance. These include pharmaceutical and toxicologic tractability in large animals and, ultimately, humans. In this respect, molecularly targeted drug screening strategies provide an efficient means of "tracking" the effect of a drug on its target throughout its development, and ascertaining that even early clinical trials are achieving results that are tied to action on the target. The best recent example of this is the proteasome inhibitor bortezomib, where development of a proteasome inhibition assay informed the choice of clinical schedule and dose escalation to allow maximal target inhibition while refraining from toxic dose ranges (41).

Numerous criteria can be put forth for a good screening assay. In cell-free assays it is critical that an adequate supply of well-characterized reagents is available. Ideally, one would like to pursue assay development and conduct screening campaigns with a single batch of reagents. When this is not possible, adequate criteria for qualification of batches must be available. For cell-based assays, it is essential that the engineered or otherwise manipulated cell populations be amenable to growth and cryopreservation. Cell banks must be carefully quality controlled, including documentation of identity (via DNA fingerprinting and other modern means) and freedom from pathogens and adventitious agents. Assay reproducibility is a key issue that can be addressed initially by retesting of small sample libraries and in the longer run by inclusion of standards and positive controls. In microplate assays these are best built into screening plates as on-board controls. Signal-to-noise ratio in screening assays is important as is reproducibility across well within plates. A formula for combining these issues to calculate an assay quality parameter known as Z' has come into wide use (42).

Conclusion

The coming decades will see the definition of detailed pathways governing processes critical for cancer cell growth, including DNA repair, cell cycle progression, and regulation of tumor-host interactions, to consider but a few. Academic investigators will make a critical contribution to the definition of new targets, even as the

commercial sector will increase the efficiency with which these targets are explored as a basis for useful drugs. Technology will evolve to refine screening and molecular recognition approaches. A continuing dialog between cancer biologists, screeners, and pharmaceutical scientists will ensure that the best opportunities are being addressed and are essential to the design of valuable screening campaigns.

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Animal Models to Predict Anticancer Efficacy

The optimal way to identify entities that have significant clinical utility as anticancer agents has been debated for almost 50 years. One would anticipate this discussion being extended until curative therapies are found for all human cancers, although it is unlikely that a single model, or approach to developing preclinical models *in vivo* will fulfill this expectation. Rather, multiple models, each with their respective strengths, will be required. Clearly, as the genetic alterations in human cancers are identified it will be important to have models that accurately recapitulate these for developing agents targeted to putative oncogenic-drivers. However, small-molecule inhibitors of receptor tyrosine kinases, or other targets, are still susceptible to resistance mechanisms that thwart current cytotoxic therapeutics. Thus, having models that realistically represent human cancer, with its associated genomic plasticity, are essential for defining the value of an agent at a preclinical level. Interestingly, two analyses of current models and their value in drug development have independently proposed very similar strategies for incorporating xenogeneic and transgenic models into screening programs (Figure 45-1; 1,2).

In the search for novel cancer therapeutics, animal models have been used as the front line in predicting efficacy and finding toxicities before entering the clinic. Different rationales to target the same goal have resulted in many different animal models of disease. The bulk of these models can be separated into two groups, grafts of tumor material (syngeneic or xenogeneic) into immune-competent or immune-deficient animals and genetically engineered animals that recapitulate a specific cancer genotype. While these two groups both possess unique qualities, the usefulness of genetically modified animals for identifying novel compounds that subsequently demonstrate significant clinical activity against the appropriate histology remains to be shown. The schema for testing agents (Figure 45-1) against models of pediatric cancers adopted by the Pediatric Preclinical Testing Program (PPTP) is based on successful preclinical testing that has identified clinically active agents both retrospectively, and prospectively. That is, agents known to be active in childhood cancer were active against the appropriate preclinical models, and novel agents identified in the models were active in the clinical histology when tested in phase 1 or 2 clinical trials.

The earliest attempt at an animal model of disease to predict efficacy, was grafts of mouse tumors into syngeneic hosts. Perhaps the greatest value of these models was to define the fundamental role of combination chemotherapy to prevent emergence of resistance, and in developing rational strategies to treat recurrent central nervous system (CNS) leukemia. These models initially were composed of murine leukemias transplanted in the subcutaneous space, which resulted in a lymphoid-like solid tumor (3). Inoculated into the peritoneum, the two primary leukemias used were L1210 and P388. Curative therapy of L1210 leukemia required lipophilic drugs that penetrated the CNS, preventing relapse, and P388 leukemia was a very sensitive screen for identifying natural products with anticancer activity. The murine solid tumors colon 38, B16 melanoma, Lewis lung and M5076 reticulosarcoma were added later. The value of these murine tumors in selecting agents to advance into the clinic was questionable. The models presented relatively limited diversity of tumor types and exhibited extremely fast growth rates. The National Cancer Institute (NCI) used these (and later added xenograft models MX-1 and LX-1) from the mid 1950s until the early 1980s for the screening of novel compounds, identifying the cytotoxic drugs that make up the bulk of our classic therapeutic armamentarium (4).

The discovery of nude athymic (nu/nu) mice that were T-cell deficient (5), and later B-cell and T-cell deficient CB17 severe combined immunodeficient (SCID) mice (6), allowed the transplantation of human tumor (xenografts) into mice. These mouse strains allow established *in vitro* human cell lines to be propagated subcutaneously reconstituting solid tumors. Human tumor tissue explants obtained from biopsy or autopsy may also be transplanted directly into these strains of mice. This ability to implant and subsequently propagate primary tumor tissue is one contribution of the immunocompromised animal strains. Molecular profiling studies indicate the retention of expression characteristics and genomic alterations when human tumors are propagated in mice. Using athymic nude and SCID mice, it has been possible to establish xenograft lines that are representative of a great many individual pathologies, thus overcoming one of the concerns of syngeneic models, the limited diversity of tumor histologies (4). The predictive value of these models has been debated

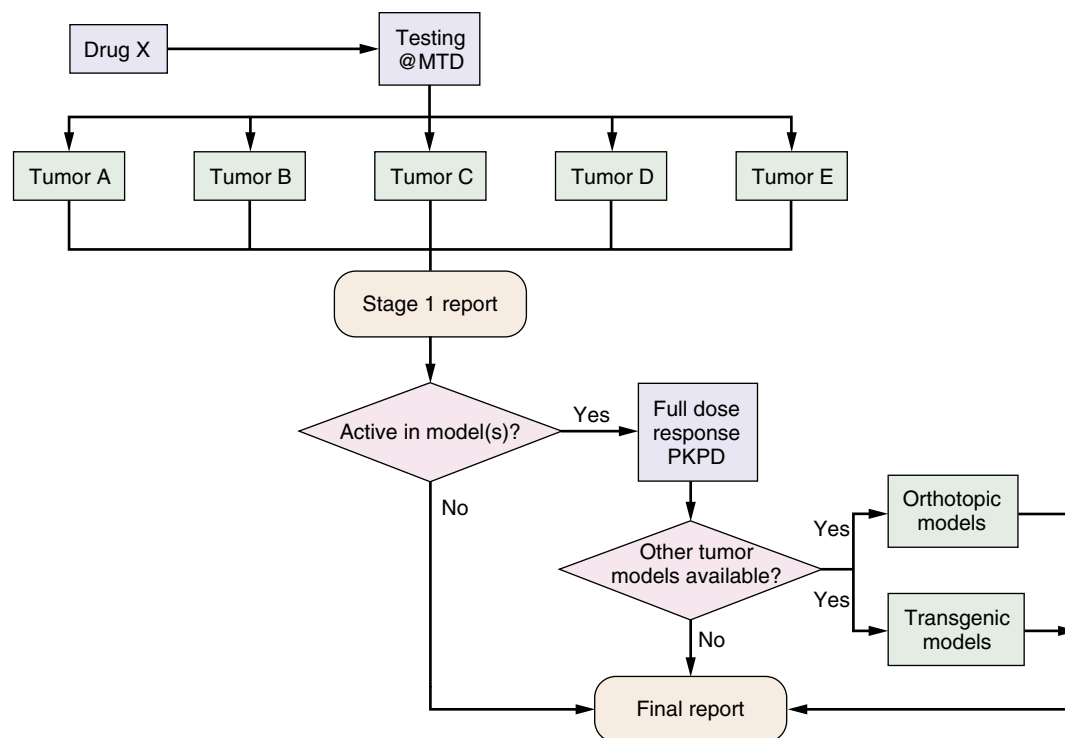


FIGURE 45-1 The testing schema adopted by the Pediatric Preclinical Testing Program (PPTP) to identify agents that may have significant activity against childhood cancers. The PPTP incorporates panels of tumors representing a particular histology (E: for example comprises six neuroblastoma xenografts). Drugs are screened anonymously (coded) at the maximum tolerated dose, or at a biologically defined dose (noncytotoxic agents), and antitumor activity defined using clinical criteria (objective regressions for cytotoxic agents) or event-free survival (EFS) for cytostatic agents (stage 1 testing). Secondary testing (stage 2) incorporates dose response studies as well as pharmacokinetic (PK) and pharmacodynamic (PD) determinations. Orthotopic models, and where available, genetically engineered models will be used in stage 2 testing. Testing data is linked to databases containing expression and single-nucleotide polymorphism (SNP) profiling for all tumors. (From Ref. 2, with permission.)

extensively. In the NCI screening program, activity of an agent in vitro did not predict for activity in either hollow-fiber assays in vivo or subcutaneous xenograft models, which in turn did not predict for activity against clinical cancer (7). However, activity of an agent against breast cancer xenografts correlated with clinical activity for the agent against one or more cancers. Further, activity in 30% of tumor models predicted for activity in some clinical cancer. However, why the models failed to accurately predict activity in the appropriate clinical histology is of concern. Goldin et al. (8) originally proposed using panels of human tumor xenografts, but resource constraints prevented development of such a screen. Further, no pharmacokinetics studies were incorporated into the NCI screening program; hence, it was not possible to retrospectively determine whether activity in the mouse model was achieved at clinically attainable drug systemic exposures. In contrast to the NCI experience, retrospective analysis showed that xenograft models, but not allografts of murine tumors, can be useful for predicting the phase 2 clinical trial performance (at least for ovarian and non-small cell lung cancer) when panels of tumors were used (9). Other studies have found the predictive value of xenograft models to be variable (10,11). For childhood cancers, xenograft models have been quite accurate in identifying clinically active agents and effective drug combinations, particularly when differences in drug exposure between tested animals and humans are taken into account (12).

One concern is how many tumors of a particular histology are required to simulate a clinical population to obtain a reasonable estimate of response rates. Our data would suggest simulating a

phase 2 trial using 12 preclinical models of a particular histology accurately identifies agents that are active against the clinical disease (13). As shown in Figure 45-2, a panel of 12 rhabdomyosarcoma xenografts were established by direct transplantation to mice, and evaluated for their sensitivity to standard agents known to have significant activity against this disease in children. Importantly, “clinical” criteria, (complete or partial response, stable disease, or progressive disease) were used to assess drug efficacy. The objective response rate to standard agents, vincristine and cyclophosphamide, was 42% and 50%, respectively, whereas that for actinomycin D was somewhat less, consistent with reported clinical data for single agents. Again consistent with clinical results, melphalan was highly active against the panel. The model also identified the camptothecin derivatives, topotecan and irinotecan, as highly active, and this activity has been confirmed in several phase 2 clinical trials. The activity of the acylfulvine derivative (MGI-114) in the model is an example of a “false positive,” as systemic exposures in mice that induce objective responses far exceed exposures in patients at the maximal tolerated dose in phase 1 trials. Relating antitumor activity to drug exposures in mice relative to exposures in patients is a critical component for models to accurately predict for human efficacy. Based on these and similar results, the NCI has supported the PPTP experiment. Drugs are evaluated against a panel of 44 pediatric cancers representing sarcomas, neuroblastoma, brain tumors, kidney cancers, and acute lymphoblastic leukemias. As a validation test two “standard” cytotoxic agents, vincristine and cyclophosphamide, were evaluated in a “blinded” manner to see

Tumor Line/Histology	Rh10	Rh12	Rh18	Rh28	Rh30	Rh35	Rh36	Rh39	IRS56	IRS68	Rh65	Rh66
Drug	ARMS	ERMS	ERMS	ARMS	ARMS		ERMS		ERMS	ERMS	ARMS	ARMS
Vincristine												
Cytoxan												
Dactinomycin												
Topotecan												
CPT-11												
L-PAM												
MG1-114												
Response key		MCR		CR	PR		SD		PD		No data	

FIGURE 45-2 The responsiveness of a panel of 12 rhabdomyosarcoma xenografts to standard cytotoxic agents used for treatment of rhabdomyosarcoma (vincristine, cyclophosphamide, and actinomycin D) and several experimental agents (melphalan [LPAM], topotecan, irinotecan [CPT-11], and MGI-114). Response rates for standard agents in the model are consistent with clinical response rates for single agents tested against rhabdomyosarcoma.

if the panels identified this agent as active. A data summary for vincristine is shown in Figure 45-3, as a “COMPARE”-type plot, where sensitivity relative to a midpoint activity (stable disease) is plotted for the response of groups of ten mice per treatment group. The objective response rate was 39% for solid tumors and all of the eight acute lymphocytic leukemia (ALL) models responded, suggesting good concordance with expectations for activity of this agent against clinical disease.

The primary transplant site of human tumor in these models is the subcutaneous route, but other tissue-specific or “orthotopic” transplant sites are possible. The advantage to using a subcutaneous transplant site is the ease of transplant as well as tumor growth monitoring, which may be done by palpation and external measuring. The disadvantage to the subcutaneous transplant site is that it does not recapitulate the natural site that a particular tumor was derived from. The obvious example where the subcutaneous

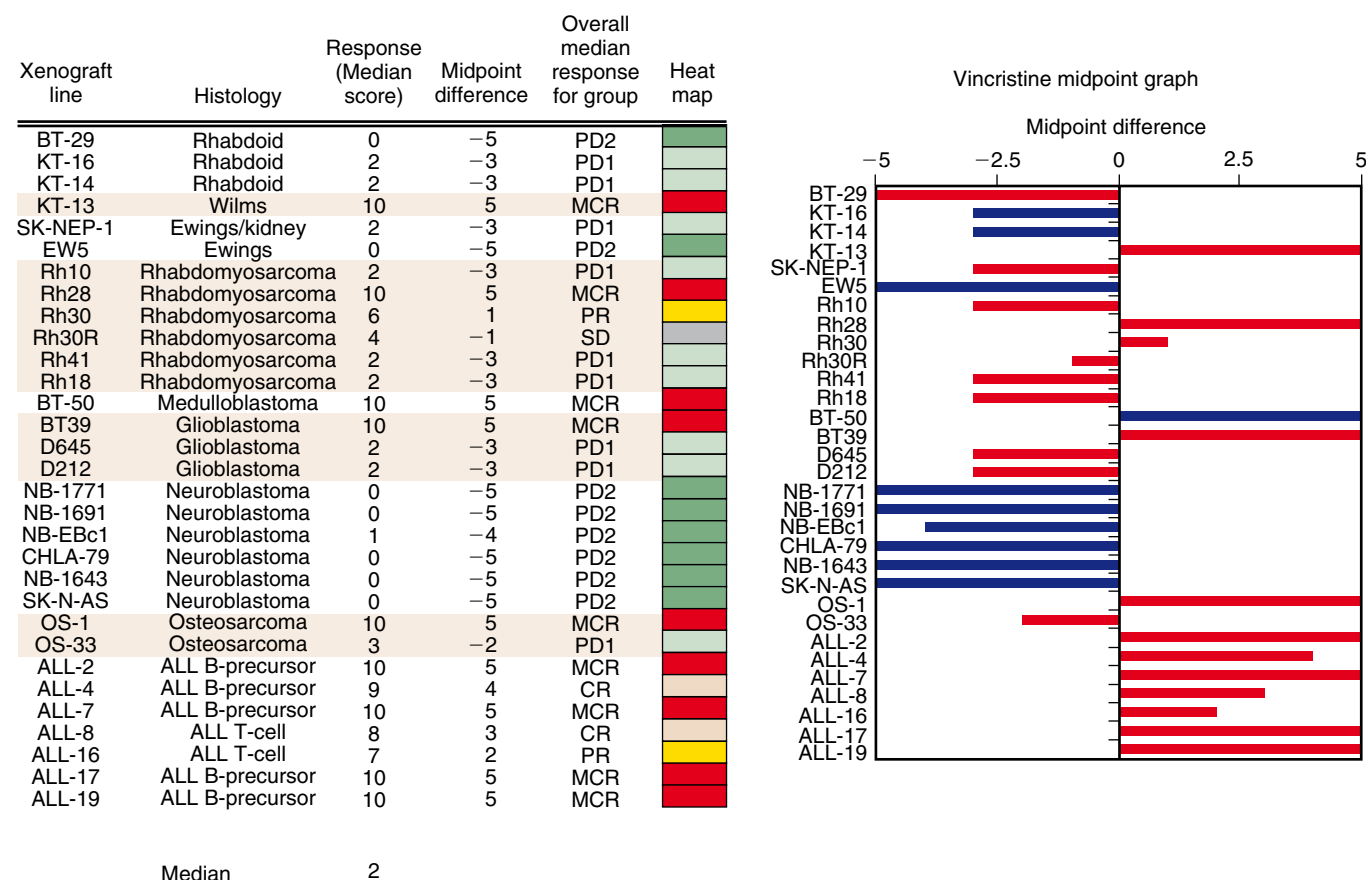


FIGURE 45-3 DATA SUMMARY FOR VINCRIStINE ACTIVITY IN THE PEDIATRIC PRECLINICAL TESTING PROGRAM (PPTP) SCREEN. Left: The colored “heat map” depicts group response scores. A high level of activity is indicated by a score of 6 or more, intermediate activity by a score of > 2 but < 6, and low activity by a score of < 2. Right: Representation of tumor sensitivity based on the difference of individual tumor lines from the midpoint response (stable disease). Bars to the right of the median represent lines that are more sensitive, and to the left are tumor models that are less sensitive.

site may yield incorrect results is with brain tumor xenografts, where drug penetration to the orthotopic site may be compromised by the blood brain barrier which would exclude or reduce drug penetration. Thus, as shown in Figure 45-3 the response of BT-39 (glioblastoma) and BT-50 (medulloblastoma) xenografts may overpredict the activity of vincristine. The question is whether the subcutaneous transplant, with its technical ease, or the more natural orthotopic transplant, is better at predicting clinical efficacy.

Orthotopic models have been developed for many different cancer types, but in some cases they are limited by the ease of implanting in a specific site. Models of adult pancreatic cancer have been established by implanting a small tumor fragment directly on the pancreas of nude mice (14). Injection of colon carcinoma cells to the spleen leads to hepatic metastases, and intracerebral tumor models of glioblastoma have been developed by injecting a tumor cell suspension through a burr hole in the skull of a CB17 scid/scid mouse (15) or athymic nude mice (16). Models of breast carcinoma have been developed by implanting a small piece of tumor in a pocket in the mammary fat pad of a nude mouse (17). Orthotopic or disseminated models of pediatric neuroblastoma have been developed through injection of a suspension of neuroblastoma cells into the retroperitoneum, adrenal gland or the lateral tail vein, which results in abdominal tumors, much like that seen in the presenting patients (18).

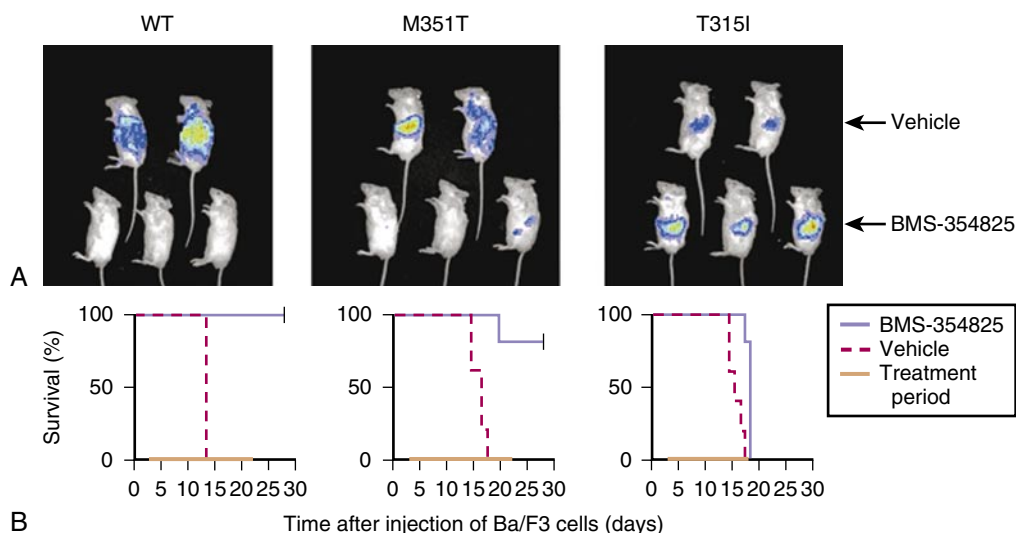
With each of these models, and the many others that have been developed, there is one difficult problem to overcome for using the model to monitor agent efficacy: How to monitor the tumor's progression. Endpoint type studies that measure survival are the only type study that can be undertaken in most cases. With the arrival of new imaging technologies, such as the Xenogen biophotonic imaging and the VisualSonics ultrasonic imaging, this problem is lessening. Examples of use of the Xenogen system for monitoring growth of hematopoietic malignancies and effectiveness of treatment are shown in Figure 45-4. The resolution for monitoring growth of liver metastases by ultrasound is shown in

Figure 45-5. There are limitations to each of these technologies as well. The Xenogen system requires the expression of luciferase in the tumor model before it can be visualized (19) and thus tends to be limited to use with cell lines, rather than with primary tissue models. The transfection or transduction of the luciferase gene into the tumor line is labor intensive and alters the tumor material. Further, the models so developed tend to be clonal, hence no longer represent the heterogeneity of the original tumor. The VisualSonics does not require any changes to the tumor line but, it also has limitations as ultrasound is unable to penetrate skull, making intracranial tumor models difficult to follow. This can only be accomplished with the technically difficult, cranial window.¹

In addition to the two previous host animals, a third immunocompromised strain, the nonobese diabetic (NOD) SCID mouse, allows for an orthotopic-type model of leukemia. Leukemias of different histologies including models of adult acute myelocytic leukemia (AML) and pediatric ALL have been successfully propagated in this host animal (20–22). Direct biopsy material is introduced into the peripheral blood through the tail vein of NOD/SCID mice and engraftment is achieved. Monitoring of the xenograft is similarly difficult and labor intensive as other orthotopic models of disease. The mice must be bled weekly and analyzed by flow cytometry using an appropriate cell surface marker such as human CD45. A continuously propagated xenograft may be maintained by harvesting the spleen of engrafted mice and enriching for mononuclear cells which can be used for a subsequent transplant (20,21). In addition to normal monitoring for animal well-being, this model must also be monitored for development of thymomas.

Genetic manipulation of the mouse genome has revolutionized development of animal models of human disease. Deletion of tumor suppressors (*p53*, *PTEN*, *RB*, etc.) yields models having reasonable penetrance, although onset of tumorigenesis may take several months, thus precluding the use of such models in high-throughput tumor-treatment strategies for drug testing. However, the potential for prevention studies remains high. Similarly, knock-in technology has led to development of numerous

FIGURE 45-4 Use of the Xenogen system for monitoring tumor responses in mice in a model of BCR-ABL-dependent hematopoietic disease. **A:** In vivo assay of growth inhibition of imatinib-resistant mutant BCR-ABL-expressing Ba/F3 cells. SCID mice were treated with a 50:50 mixture of propylene glycol and water (Vehicle) or BMS-354825, beginning 3 days after infusion of the Ba/F3 cells. Images were obtained after luciferin injection on day 13. Luciferase activity was primarily detected in the spleen. **B:** Kaplan-Meier survival analysis of BMS-354825-treated SCID mice harboring BCR-ABL-WT, M351T, and T315I isoforms. (From Ref. 36, with permission.)



¹ Personal communication, Tim Shannon, VisualSonics.

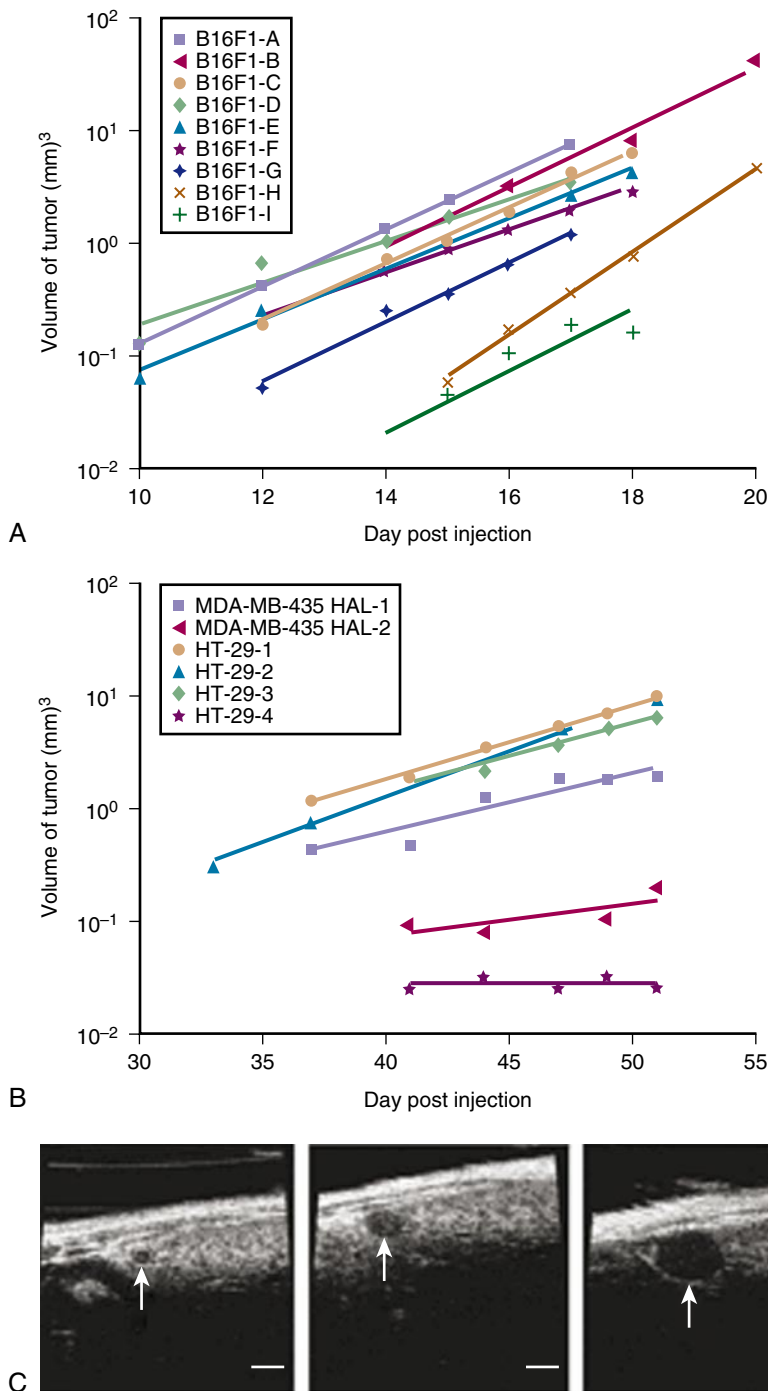


FIGURE 45-5 Tracking the growth of individual liver metastases by noninvasive ultrasound imaging. **A:** Growth curves of B16F1 liver metastases. **B:** Growth curves of HT-29 (colon adenocarcinoma) and MDA-MB-435/HAL (breast carcinoma) liver metastases. **C:** Representative two-dimensional ultrasound images of B16F1. (Bar on the ultrasound images = 1 mm). (From Ref. 37, with permission.)

cancer models through over-expressing oncogenes such as Ras, cyclin D1 or MYCN, or compound mutations found in clinical cancer (23). Backcrossing different models, for example the *ptc*^{-/-} model of medulloblastoma on a p53-null strain yields a compound genetic model with high penetrance (24) and more rapid onset. Compound genetic models while perhaps more readily recapitulating human cancer, can yield some unanticipated results. For example, overexpression of hepatocyte growth factor/scatter factor (HGF/SF) in *Ink4a/Arf*^{-/-} mice, originally conceived to be a model of human melanoma, yields multifocal rhabdomyosarcoma

(25). Thus, one has to be concerned that the “correct” genetics are associated with the appropriate human histology, if conventional drug development approaches are taken, where the assumption is that models of a particular tumor type predict for responses of that clinical histiotype.

Although the potential for genetically engineered models for cancer drug development is high (26), currently there is no clear data, to support the value of these models over other less demanding models. Rather, at this time the predominant value of these models appears to be in understanding the biology of cancer. There

are, however, compelling characteristics that indicate that these models may be valuable in drug development. Tumors arise spontaneously at their natural site, thus overcoming some of the concerns raised for xenogeneic models. Tumors arise in immune competent animals and hence have an advantage that the models can be used to develop effective immunologic therapeutic strategies. The prototypic model, the so-called Onco-mouse model of breast cancer, is driven through oncogenic v.Ha-ras-targeted to mammary tissue through use of the viral MMTV-promoter. This model was used extensively in development of farnesyltransferase inhibitors (FTIs; 27), which induced sustained regressions. While FTIs have activity in clinical cancer, particularly against certain leukemias, the mechanism (inhibition of Ha-Ras) remains controversial as many cellular proteins require farnesylation. Tissue-specific promoters have been used extensively to construct models of breast hyperplasia and cancer (28). Other rare tumors, for example neuroblastoma, where MYCN is driven from a tyrosine hydroxylase promoter (29), or myf6-driven Pax3/FKHR induces rhabdomyosarcoma (30) have been constructed, although there is limited use reported for drug development, other than a proof of principle experiment where MYCN antisense was used in vivo to retard development of the neuroblastoma model (31). The value of conditional transgenic models such as the doxycycline-induced, bitransgenic MMTV-rtTA/TetO-NeuNT model of multiple invasive mammary carcinomas (32) also remains to be defined in the context of drug development. When the transgene is de-induced even metastatic tumors show massive involution, but mice succumb with recurrent neu-independent tumors. Such models may recapitulate the activity of herceptin (an antibody targeted to the Her2/neu receptor) in human breast cancer. Whether the recurrent tumors recapitulate postulated mechanism(s) of herceptin resistance in human cancer (for example, increased signaling through the IGF-I receptor) remains to be determined. An alternative approach to creating compound genetic models of human cancer is through use

of avian retrovirus gene delivery. In a model of ovarian carcinoma, ovarian cells from transgenic mice engineered to express the avian retroviral receptor, TVA, are efficiently infected with multiple vectors carrying oncogenic coding sequences. In ovarian cells from p53-null mice, the addition of any two oncogenes (c-myc, K-ras, or Akt) induced tumors when innoculated into the subcutaneous, intraperitoneal, or ovarian sites (33). Further development of this model to allow conditional expression of the oncogene has been reported (34) although its use in drug development has not.

Cancer therapy is moving from an era of empiricism to one where potentially treatment strategies will be defined by tumor genotype or other molecular characteristics. For effective drug discovery and development it will be imperative that genetic changes involved in transformation of human cancers are accurately recapitulated in the preclinical models. This applies for development of specific inhibitors, as well as identification of active agents in high-throughput screening programs. With current technology it is possible to construct genetically engineered models with compound genetics representing specific histologies and their subtypes. Detailed molecular characterization of available human tumor models will be essential to identify appropriate preclinical screens for development of molecularly targeted therapeutics. For each of the models, close attention to potential differences in pharmacology and toxicity between mice and humans remains a major factor preventing accurate translation of preclinical results to treatment of clinical cancer.

Acknowledgments

Original work presented here was supported through USPHS grants CA23099, CA96696, and CCA21675 (Cancer Center Support Grant), and NO1CM42216 from the National Cancer Institute.

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Pharmacokinetics, Pharmacodynamics, and Pharmacogenetics¹

Cancer is a difficult set of diseases to treat, so it is essential to maximize the benefit that can be obtained from each therapeutic agent. There is a compelling rationale to match the choice of drug to the tumor and the patient, to the extent feasible. In addition to selection of therapy, we also need to have tools to assure that the *delivery* of therapy and the *evaluation* of a drug's impact on its target are properly managed. Our major investments in translational research will be stymied without sufficient attention to these important issues.

The narrow therapeutic index of anticancer drugs is a powerful motivation for the careful control of drug delivery, even for the average patient. Of course, for the era of personalized medicine, our attention is oriented towards the issue of variability within a population, and we are also focused upon the characteristics of individual patients.

Within pharmacology, pharmacokinetics (PK), pharmacodynamics (PD), and pharmacogenetics (PG) have their separate aspects, but they are highly-interrelated (Figure 46-1 and Table 46-1). Any consideration of variability in either PK or PD parameters leads to consideration of PG.

Pharmacokinetics

The world of PK can be enshrouded in equations, but it is preferable to focus upon the concepts that are needed to gain the potential benefits of PK. These benefits are directed toward answering therapeutic questions both for the average patient and for individuals that differ from the average.

In the overwhelming majority of cases, drugs are prescribed for an average dose. The process of drug development defines the

Table 46-1 Definitions

Pharmacokinetics (PK): What body does to drugs (drug delivery)
Pharmacodynamics (PD): What drugs do to the body (drug action)
Pharmacogenetics (PG): Heritable traits related to drug delivery and drug action

important dosing parameters for the population of patients that will receive the drug. When a protocol is designed for an investigational agent, answers are sought for a specific population. The emphasis is nearly always upon the average patient. Subsequently, as a drug is used in broader populations, vigilance is required to observe individual variations in PK and to adjust accordingly.

Why Should We Be Interested in Pharmacokinetic Concepts?

The easiest way to appreciate PK concepts is to examine the questions that they address. To obtain answers to the questions raised in the treatment of both populations and individuals, it can be helpful to describe a few underlying PK concepts.

As summarized in Table 46-2, clearance, half-life, and bio-availability can be useful PK parameters for nearly all therapeutic situations. Of course, there are additional PK concepts that may be important in specialized cases, including volume of distribution and transport rates between tumor and plasma.

Clearance

Clearance is the parameter that determines total systemic exposure to drug, which is simply the ratio of dose/clearance. Total body clearance is the sum of all processes by which drugs are removed from the body or inactivated, primarily renal excretion and metabolism.

The primary application of clearance is for dose adjustment in patients at extreme ends of population (i.e., those that have high or low clearance relative to the average value for the population).

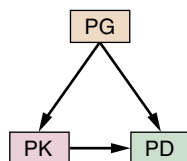


FIGURE 46-1 Genetic control of enzymes, receptors and other factors related to pharmacokinetics and pharmacodynamics.

¹ "All material in this chapter is in the public domain, with the exception of any borrowed figures or tables."

Table 46-2 Linkages Among PK Concepts and Therapeutic Goals

Therapeutic Question	Answer Sought	Relevant PK Concept
(1) How Much?	Dose	Clearance
(2) How Often?	q.d; b.i.d.	Half-Life
(3) What Route?	i.v.; p.o.	Bioavailability
(4) Drug-Drug Interactions?	concomitant use OK	Metabolism

b.i.d., twice daily; IV, intravenous; p.o., by mouth; q.d., daily.

Low Clearance→High Systemic Exposure
High Clearance→Low Systemic Exposure

Thus, adverse drug events, which can be related frequently to overexposure, would be expected more often in patients with low clearance. Similarly, even when the drug is ideally matched to the tumor characteristics, failure to attain therapeutic benefit can be related to under-exposure in patients with unusually high clearance.

Systemic drug exposure is an index of drug delivery to the tumor and normal host tissues. Transport processes, such as efflux pumps and intracellular metabolism, may affect the ratio of tissue exposure to systemic exposure. In such cases, systemic exposure may not be directly useful in determining tissue exposure, but clearance remains useful as an index to track changes in drug delivery to those transport processes.

Half-Life

The time that a drug stays in the body is quantified as its half-life. The half-life for anticancer agents ranges from a few minutes for some alkylating agents and antimetabolites, to several days or weeks for monoclonal antibodies.

As we continue to shift the emphasis in anticancer therapy from intermittent dosing toward chronic therapy, especially on a daily basis, half-life helps us to determine the dosing frequency. Not all drugs require continuous exposure above some threshold concentration in plasma, but that is frequently the starting point for the design of therapeutic regimens.

If the half-life is at least 12 hours, then dosing once per day is often feasible, as for imatinib. However, if the half-life is a few hours or less, multiple doses per day may be necessary, as for capecitabine. If the half-life is a few weeks, then monthly dosing may be appropriate, and the practical advantage of oral delivery is less impressive.

Bioavailability

As research produces an increasing number of drugs that are intended for continuous use rather than intermittent delivery, the practical advantages of oral dosing become clear. Consider the development of imatinib, the most successful of recently approved anticancer agents. Although the usual spotlight is understandably placed on the specificity of imatinib’s targets in chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor (GIST), those outstanding properties would be worthless unless imatinib can be given continuously. If the intravenous (IV) route was the only option for imatinib, it would not be a successful drug.

Fortunately, the bioavailability of imatinib is 98%, which means that the systemic exposure generated by a 400-mg tablet is 98% of that generated by 400 mg IV.

Metabolism

In addition to its role as a contributor to total body clearance, metabolism is important in its own right. The most common drug–drug interactions are produced by decreases or increases in the rate of enzymatic metabolism of one drug by a second drug. Knowledge of both the pharmacogenetics of the drug-metabolizing enzymes and the inhibitory or induction potency of drugs permits many of these interactions to be predicted in advance, with the opportunity to avoid either underexposure or overexposure.

Metabolism can create molecules that are more active than the parent drug. For prodrugs such as cyclophosphamide, the therapeutic activity depends on metabolic activation. In general, metabolites can also be more toxic than the parent drug, but these cases tend to be so complex that the parent drug is abandoned.

In the early stages of drug development, interspecies differences in metabolism can confound the relation between preclinical and clinical findings. For example, the principal metabolite of paclitaxel in humans is not formed in rats (Figure 46-2; 1). In this case, peak “H” does not have substantial pharmacologic activity, but the potential for increased paclitaxel toxicity if metabolism were inhibited is different between rat and human. Similarly, there is a difference between rats and humans in the potential for reduced paclitaxel antitumor activity due to induction of metabolism.

Pharmacokinetics: Benefits for Individual Patients

Most of the preceding discussion focused on getting a single answer to important questions about drug therapy in a particular population. If all the PK parameters of a drug were the same in

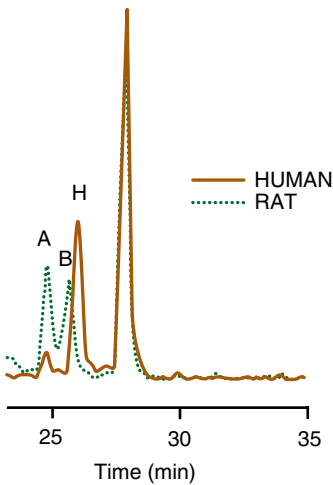


FIGURE 46-2 Comparison of metabolite profile for paclitaxel by high-performance liquid chromatography. The major human metabolite, peak “H,” is not formed in rats. (From Jamis-Dow CA, Klecker RW, Katki AG, Collins JM. Metabolism of taxol by human and rat liver in vitro: a screen for drug interactions and interspecies differences. *Cancer Chemother Pharmacol* 1995;36:107–114, with permission.)

Table 46-3 Major Sources of Variation in PK in a Population

Renal or hepatic impairment
Polymorphic metabolism
Environmental influences

all patients, PK would be very straightforward. Clearly, not all patients are the same, and the individualization or customization of therapy is necessary to consider for patients whose PK properties differ from the “average” patient. We have always been aware of individual differences, and our tools for understanding the basis and predicting the phenotypic consequences are rapidly improving. The major sources of variation for PK are listed in [Table 46-3](#).

For drugs that are primarily excreted by the kidneys, wide variation in renal function among individual patients drives adjustments from the standard or average dose. Carboplatin dosing based on creatinine clearance will be discussed in the next section. Hepatic impairment is an established risk factor that often requires dosage reduction, but the appropriate index for degree of impairment is poorly documented. Thus, although creatinine clearance serves as a guide for renal function for many drugs, there is no universal test metabolic variation.

For drugs that are primarily inactivated by metabolism, it is important to recognize that the expression of each enzyme is separately influenced by pharmacogenetics. Polymorphisms generally decrease the catabolic activity of enzymes, leading to potential decreases in doses. However, there are cases in which mutant alleles are more active than the most common allele, leading to reduced systemic exposure unless there are compensatory increases in dose.

The term “environmental influences” includes variation in diet or exposure to substances that injure normal physiology. Various food products can either increase or decrease enzyme activity. Concomitant therapy or co-existing disease can change either PK or PD.

Pharmacodynamics

Arguably, the most important part of pharmacology is PD, what the drug does to the body. Regrettably, PD is also the least understood. The determinants of drug action are linked to both the pattern of drug delivery and the specific details of the interaction of the drug and its receptor.

What is the optimal way to deliver a drug such that antitumor effect is maximized while toxicity in normal tissues is minimized? PK is a delivery engine that can provide various patterns of exposure for a short bolus (brief pulse), continuous infusion, or intermittent exposure.

Traditionally, PD is viewed as a dose-response curve, or preferably as a concentration-response curve, so that the link to PK is established. The link is only a start, because the fundamental question links response to both time and concentration.

PD covers both desired effects (antitumor activity) and adverse effects (toxicity). Ironically, although our highest motivation is the achievement of efficacy, the most definitive studies for

the PD of anticancer agents are relationships between normal tissue toxicity and dose or concentration. The overarching goal, of course, is to find differential effects on tumors and normal tissue.

The merit of an approach of linking PD to PK for toxicity reduction was demonstrated in the 1970s when high-dose methotrexate (MTX) regimens produced a large range of toxicity in patients, with no obvious prognostic factor. It was found that those patients who developed the most severe and prolonged toxicity were those with delayed removal of MTX from plasma. Thus, target concentrations for avoiding toxicity were developed and concentrations monitored in patients. The practical outcome was selection of which patients needed to be hospitalized and treated more intensely with leucovorin and which could be sent home with reasonable assurance that their toxicity would be within expected limits. This strategy was generally successful; however, the “safe target concentrations” were not universal, but depended on the specific MTX dose and schedule that were used. In that era, there were many different regimens tested in attempts to improve anti-tumor response, a worthy goal but with confounding of efforts to identify patients at higher risk of severe toxicity.

A decade later, Egorin et al. (2) demonstrated the value of plotting an index of relative toxicity on the y-axis versus drug exposure on the x-axis emerged. As shown in [Figure 46-3](#), the percentage change in platelets could be related to total area under the curve (AUC) of carboplatin, which could be readily estimated from creatinine clearance. This type of graphical approach rapidly became the standard for phase 1 clinical trials,

The information from this figure was not just collected as a passive observation of biologic phenomena, but was used in combination with PK data to provide an active approach to individualized dosage selection, as described in [Table 46-4](#).

Plasma concentration does not always reflect exposure at the tumor site. Nonetheless, relationships such as those in [Figure 46-3](#) have made plasma concentration the most frequently-used biomarker (or surrogate) for drug effect.

Increased emphasis on oral delivery gives us powerful options for continuous delivery that have been mostly impractical via other routes. With more options, more questions emerge. Is there some period of continuous delivery (e.g., 2 weeks), which should be alternated with intervals that are drug-free (e.g., 1 week), to permit healthy tissues to recover?

Shifting our attention from drug delivery, the PD of a drug's action is also determined by the specific properties of its target. Most drug actions are mediated via receptors such as binding proteins or enzymes. Enzyme inhibitors range from our oldest drugs (methotrexate inhibition of dihydrofolate reductase, fluorouracil inhibition of thymidylate synthase) to the large family of contemporary tyrosine kinase inhibitors. What are the factors that change drug action? Some initial things to investigate are high (or low) expression of the enzyme target, or mutations that change catalytic properties for both its substrate and its inhibitors.

We seem to be better at individualizing therapy than at defining the average delivery pattern for the population. Although our understanding of PD has lagged behind other areas of pharmacology, two sets of tools are rapidly changing our ability to acquire PD-related data. When biopsies of tumors are available for laboratory

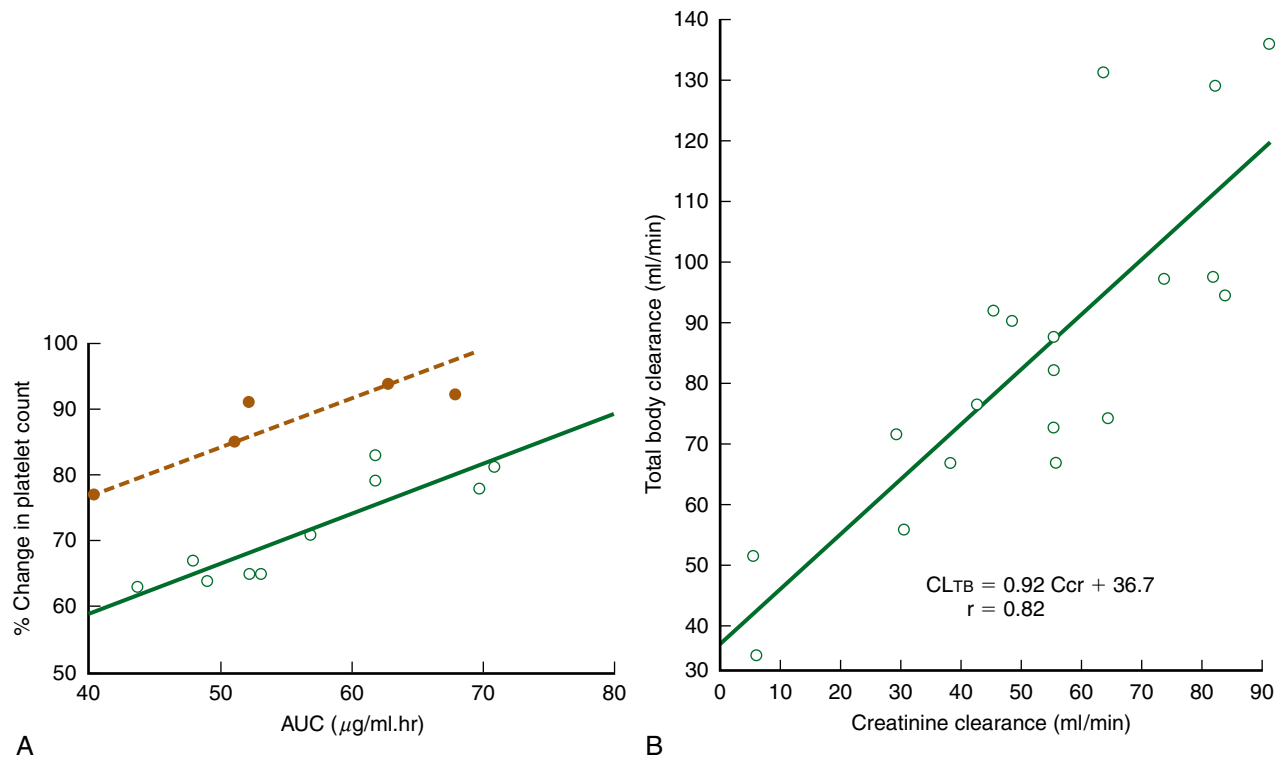


FIGURE 46-3 A: Thrombocytopenia related to carboplatin drug exposure (area under the curve [AUC]), **(B)** which was calculated from creatinine clearance. (From Egorin MJ, Van Echo DA, Tipping SJ, et al. Pharmacokinetics and dosage reduction of cis-diammine(1,1-cyclobutanedicarboxylato)platinum in patients with impaired renal function. *Cancer Res* 1984;44:5432–5438, with permission.)

studies, molecular diagnostics have provided a tantalizing hint of what can be done when response can be measured. Among other advantages, these approaches have the potential advantage of improving the PD data by screening out non-responders.

When biopsies are not feasible, noninvasive imaging has become an alternative. Positron emission tomography (PET) imaging with ¹⁸F-fluorodeoxyglucose (FDG) has broad application at finding tumors, and increasingly, we are learning when it can be used to monitor the impact of therapy. In Figure 46-4, PET images are shown for two patients who had estrogen receptor–positive primary breast tumors. Following initial therapy, both patients developed metastatic disease (3). Before treatment with hormonal therapy, FDG revealed bone metastases (second column of images). Based on clinical evidence and a second set of FDG images (third column), patient A responded favorably but patient B did not.

The first column of images shows pretreatment scans obtained with ¹⁸F-fluoroestradiol (FES), an investigational probe for estrogen receptors (ERs). Patient A has colocalization of FES with FDG, suggesting that the metastases are ER positive. The major metastasis for patient B does not have localization of FES,

suggesting that it is ER negative. Both findings are consistent with other tests of ER status. FES can determine ER status and help to guide the choice of appropriate therapy.

Pharmacogenetics

For patients with cancer, there are two genomes that require attention. The genes in the tumor overlap with the genes in the germ line. Thus, the similarities and differences must be carefully sorted out as we seek a therapeutic advantage. Figure 46-1 indicated that PG can influence of both drug delivery processes (PK) and drug target properties (PD). Figure 46-5 provides some specific examples.

The host or germ-line genetics create variation in the patient population for drug-metabolizing enzymes such as UGT1A1, a member of the uridine glucuronyl transferase superfamily that inactivates SN-38, the active species derived from irinotecan (4). About 15% of white patients have inefficient UGT1A1 alleles, with lower incidence in Asians populations and higher incidence in black populations. Similarly, a small percentage of patients are deficient in dihydropyrimidine dehydrogenase (DPDase), the enzyme that inactivates at least 80% of a fluorouracil dose.

For both of these examples, the toxicity observed in sensitive normal tissues is exaggerated compared with that of the average patient. Fortunately, reduction of doses can minimize toxicity while preserving efficacy by achieving the same systemic drug exposure experienced by the rest of the population.

In a dramatic example of the difficulty in developing a drug without knowledge of PG for drug-metabolizing enzymes,

Table 46-4 Combining PK and PD to Develop a Dosing Strategy

[1] PD: What is relationship between efficacy and drug exposure?
[2] PK: What are the determinants of drug exposure?
[3] How can the dosage be adjusted to provide similar exposures for all individuals in a population?

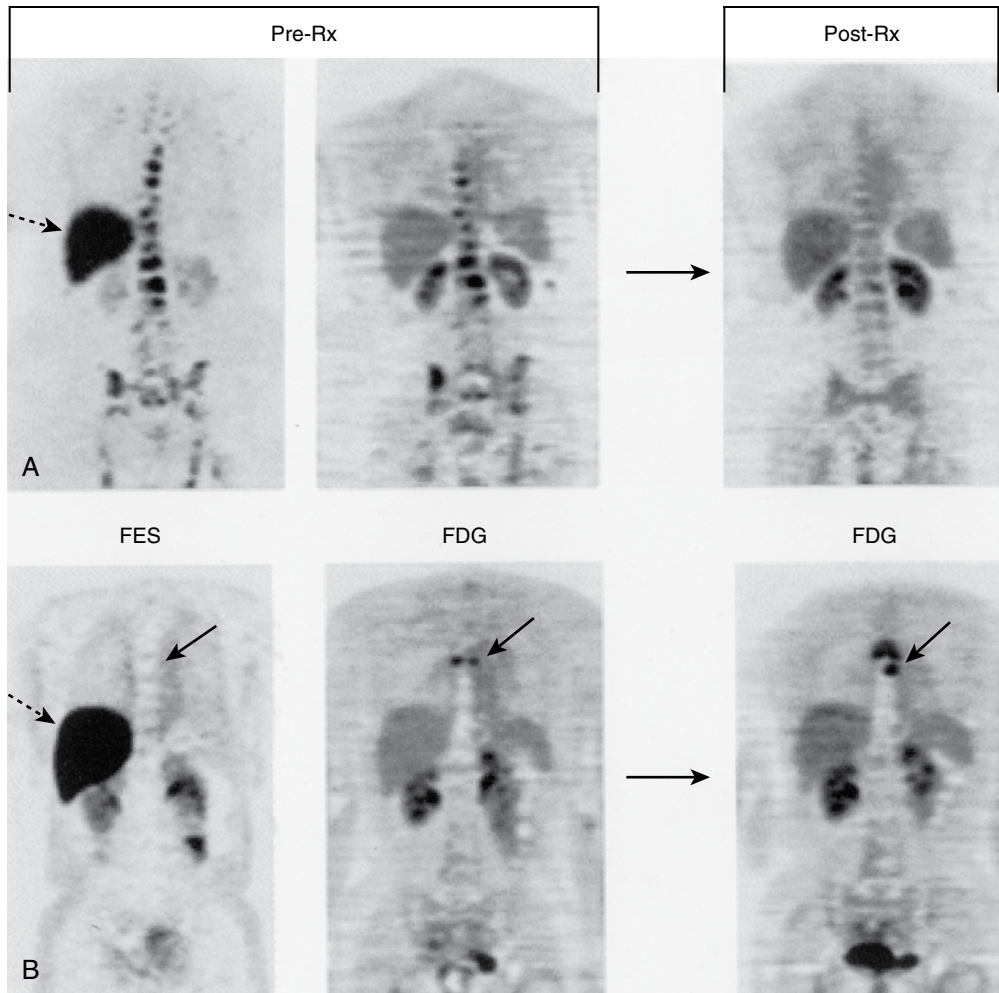


FIGURE 46-4 Positron emission tomography (PET) imaging of two patients with metastatic breast cancer before and after hormonal therapy. See text for details. FDG, ^{18}F -fluorodeoxyglucose; FES, ^{18}F -fluoroestradiol. (From Linden HM, Stekhova SA, Link JM, et al. Quantitative fluoroestradiol positron emission tomography imaging predicts response to endocrine treatment in breast cancer. *J Clin Oncol* 2006;24:2793–2799, with permission.)

amonaftide was initially found to have a maximum tolerated dose of 300 mg/m²/day. After it was discovered that *N*-acetyltransferase was the enzyme responsible for changing systemic exposure to amonaftide and its active metabolite, the tolerated doses were determined in the “slow” acetylation subpopulation to be 375 mg/m²/day, and 236 mg/m²/day in the other half of the patients. The “standard” dose of 300 mg/m²/day was not correct for either subpopulation; too low for half the population and too high for the other half (5).

In contrast to germ-line changes in PK, changes in germ-line PD can produce limits in drug exposure when normal tissues can only tolerate low exposure to drug. For example, variants of

thymidylate synthase (TS), the target for fluorouracil, limit both the dose and the systemic exposure that supplies drug to tumor. Thus, to tolerate the drug, the likelihood of antitumor response is compromised.

Within the tumor, certain variants in transport properties (PK) can influence the antitumor response. For efflux pumps in the ABC superfamily, mutations that have high intrinsic activity or high expression of activity are unfavorable findings for response to many drugs. Similarly, mutations in the tumor can affect PD. For lung cancer, mutations in the epidermal growth factor receptor, especially variant III, actually improve the antitumor response to gefitinib.

It is exciting that PG-based molecular diagnostics can pinpoint small changes in the tumor genes at the level of single-nucleotide polymorphisms. However, it is critical to keep in mind that these changes may have no clinical impact. Functional correlates of genetic differences ultimately determine their importance in therapy. A good phenotyping probe is critical for determining changes in function. Because concomitant medications can also serve as inhibitors or inducers of function (e.g., enzyme activity), these drugs can mimic genetic polymorphism. Therefore, a careful inventory of medications is useful in such studies.

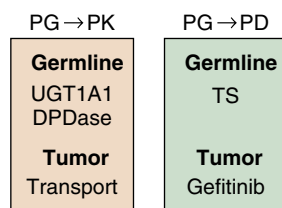


FIGURE 46-5 Examples of pharmacogenomics’ influence on pharmacokinetics and pharmacodynamics, either germ-line or tumor genome.

Conclusion

Table 46-5 provides three rules that describe drug–target interactions and summarize the roles of PK, PD, and PG. The delivery system for drugs is PK, and if the molecules do not reach the target, therapy fails. Providing sufficient drug delivery to the tumor is necessary, but not sufficient to assure antitumor response.

Variations in the tumor target receptor can also alter anti-tumor response. Adequate exposure for one tumor will be insufficient to overcome inherent resistance due to altered receptors in another tumor. PG is the major engine for variation in both PK and PD, confounding interpretation and study designs. Fortunately, the emergence of a toolbox of techniques for monitoring PG has already made a major difference in drug development. Further, these techniques are now beginning to provide instructions to pre-

Table 46-5 Three Rules for Drug-Target Interactions

If the drug doesn't reach the target, it can't have an impact. (PK rule)
Even if the drug is freely available to the target, it doesn't necessarily have the desired impact. (PD rule)
Both drug delivery (PK) and target response (PD) can be determined by genetic factors (PG)

scribers in the drug labeling that permit therapeutic adjustments for individual patients.

This chapter has been intended to focus the reader's attention on the applications of PK, PD, and PG in drug development and clinical therapeutics. More detailed descriptions of the methods of clinical pharmacology are available in print or online (6,7).

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Phase 1 Trials Today

All commercially available anticancer agents must have undergone phase 1 investigation as part of their clinical development. As the evolution of novel anticancer drugs evolved from primarily cytotoxic agents to targeted therapies, clinical investigators have developed novel Phase 1 trial designs and endpoints. It is estimated that approximately 500 anticancer agents will present to the clinical arena within the next decade. It is a well-known fact that one of the most important components of conducting phase 1 trials is eligible patient availability. As the number of commercially available agents for several tumor types has increased, as well as the number of patients treated off-protocol in community settings, the availability of patient resources has become a challenge.

As a result, it is important to conduct efficient and effective trials by maximizing data acquisition while minimizing patient numbers. Previously, standard phase 1 trials used large patient numbers and cohorts. Now, it is the norm to use well-thought-out trials minimizing patient numbers and cohorts by using alternative designs and carefully selecting the starting dose. Once thought of only as an alternative to hospice with no significant benefit, treatment on a phase 1 trial is now viewed as an additional therapeutic option. The overall clinical benefit of phase 1 trials is approximately 45%, with highly variable response rates, depending on the type of agent and the Phase I trial under investigation (1). Ethically, the intent of all clinical studies, for both the patient and physician alike, is therapeutic (2–4). A better understanding of the compound(s) under investigation and the various types of phase 1 clinical trials available will assist the investigator in determining at what point, and for which patient, specific phase 1 clinical trials should be considered.

Types of Phase I Clinical Trials

Phase I clinical trials are the first stage of drug testing in human subjects. These studies play a vital role in the development of novel therapeutics. Phase I studies are typically designed to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of a novel agent. Novel cancer therapeutics are usually offered to patients with advanced cancer who have had other types of therapy and who have few, if any, remaining treatment options. In addition,

due to several tumor types with limited current treatment options which could impact favorably on patient survival, it is considered ethical to treat patients with metastatic disease in phase 1 trials by using novel agents in combination with standard therapies; this is especially true if the standard therapies demonstrated therapeutic success in previous clinical investigations.

While the primary stated objective of a typical phase 1 study is to determine the optimal dose of a novel therapeutic for use in subsequent studies, several different types of phase 1 clinical trials exist. A brief overview is given here of several phase 1 trials that meet specific needs for clinical early drug development.

Single Ascending Dose

Single ascending dose (SAD) studies are those in which groups, or cohorts, of up to six patients are given a small dose of the drug and observed for a specific period of time. If the cohort does not exhibit any adverse side effects, a new group of patients is then given a higher dose. This continues until intolerable side effects are observed. Once such side effects occur, the cohort may be expanded to determine if more side effects are observed. If an unacceptable number of intolerable side effects occur, the dose will be lowered and tested with more patients. The highest dose administered to a patient on a phase 1 trial is referred to as the maximum administered dose. The dose that is as high as possible but still tolerable for patients is said to be the maximum tolerated dose (MTD).

Often, SAD clinical trials can be categorized as being first-in-human, first-in-class, or a combination of the two. As the name implies, first-in-human clinical trials are those that are conducted for the first time in a human patient. In order to be tested in humans, a drug typically has to first show promise of activity in the laboratory and in animals. Normally, a small (approximately 20) group of patients will be selected for inclusion into a first-in-human phase 1 study. First-in-human studies are almost always done in a single ascending dose manner. The objective of the first-in-human phase 1 trial is to find a suitable safe dose, (the MTD) for use in later studies that will more thoroughly examine efficacy. Once the MTD has been determined in a phase 1 SAD study, later-phase studies can be designed and multiple ascending dose studies can be performed.

First-in-class studies examine novel drugs that are uniquely manufactured or based on a new target or indication. Such therapeutics are typically innovative, novel, and no other pharmaceutical products are currently approved for the same therapeutic indication; hence, they have no pharmaceutical substitute.

Multiple Ascending Dose

Multiple ascending dose (MAD) studies are conducted to better understand the pharmacokinetics/pharmacodynamics of a drug. The primary purpose of a MAD study is not to determine the MTD, but rather to examine the biologic effects of the drug. In these studies, a group of patients receives a low dose of the drug and the dose is subsequently escalated up to a predetermined level. Specimens (of blood, and/or other fluids) are collected at various time points and analyzed to understand how the drug is processed within the body.

Method and Model

Method and Model (MeMo) trials are studies that are done in anticipation of a phase 1 clinical trial. Typically done for “targeted” agents, these trials help in the development of a pharmacodynamic endpoint. It may help identify either a direct tissue or a surrogate tissue marker. This assists in determining if the marker can be measured within the tissue and also helps to refine the assay needed for pharmacodynamic measurement.

Phase 1 Trials Using Radiolabeled Tracer Doses

The use of radiolabeled experimental agents has become an increasingly important factor in drug development. In preclinical studies, radiolabeled compounds are frequently used in the laboratory to understand the distribution, metabolic fate, and localization of experimental drugs both *in vitro* and *in vivo*. Clinical studies performed as part of phase 1 trials, or in support of them, may also involve the administration of small doses of radiolabeled compounds, called tracers, to healthy human volunteers or to patients to better understand the mechanisms of drug action.

Radiolabeled tracers are synthesized by replacing one or more atoms of an experimental drug agent with a radioisotope. Radioisotopes must have a suitably long half-life in order to allow for imaging or detection in biologic samples. Examples of commonly-used isotopes for detection in tissue or blood samples include carbon (^{14}C), hydrogen (^3H), sulfur (^{35}S), and iodine (^{125}I). Isotopes which are commonly used in imaging, specifically in positron emission tomography (PET) scanning, include fluorine (^{18}F), carbon (^{11}C), and oxygen (^{15}O).

Radiolabeled compounds have allowed researchers to study many aspects of a drug's behavior *in vivo* (5). Evaluation of the mass balance of a drug can be performed to better understand how much of an applied dose is recovered with respect to time. The metabolism of the drug can be extensively studied to determine if any metabolites might represent a potential toxicologic hazard to the patient. Advances in clinical imaging have greatly impacted drug discovery and development in recent years (6). Clinical imaging studies

using labeled drug have the potential to facilitate early clinical pharmacokinetic/pharmacodynamic assessments, including target interaction and modulation (7). This is particularly useful in patients where there are no direct measures of pharmacokinetics/pharmacodynamics throughout the tissues of the body and at the target.

Studies using a method called “microdosing” offer the prospect of taking a drug directly into human studies by administering extremely low doses of radiolabeled agent. Microdosing studies may also be referred to as phase 0 studies. By using only very tiny amounts of radiolabeled drug, researchers use microdosing to establish the likely pharmacologic dose and thereby determine the first dose for a subsequent phase 1 study. However, microdosing is not without controversy among researchers in drug development (5). Concern has been raised that microdosing may not accurately predict the behavior of clinical doses. It has also been suggested that nonlinearities may be induced when binding, metabolizing, or eliminating systems become saturated, thus resulting in differences between low and high doses.

Drug/Food Metabolic Interaction Studies

The U.S. Food and Drug Administration (FDA) has recommended the metabolism of an investigational new drug be defined during drug development and that interactions with other drugs be explored as part of an adequate assessment of its safety and effectiveness (8). Medicines are often used concomitantly with other drugs, and some degree of drug–drug interaction often occurs with concomitant use. Although only a small proportion of this interaction is clinically significant, it sometimes causes serious adverse reactions.

Concomitant medications can abruptly alter metabolic routes of absorption and elimination. The risk of a drug–drug interaction depends on the number of drugs used, the tendency of particular drugs to interact, and the amount of drug taken. Types of drug–drug interactions include duplication, opposition (antagonism), and alteration of what the body does to one or both drugs. Observed changes arising from metabolic drug–drug interactions can be substantial—an order of magnitude or more decrease or increase in the blood and tissue concentrations of a drug or metabolite—and can include formation of toxic metabolites or increased exposure to a toxic parent compound. In addition to potential interactions of an experimental agent with other drugs, it has long been recognized that some foods and drugs, when taken during the same period of time, can alter metabolism or cause serious adverse events.

Therefore, early on in the drug development process, appropriate efforts should be made to predict the nature and degree of potential interactions so that patients will not be adversely affected. The cytochrome P450 (CYP450) family of enzymes is an important group of enzymes found in the liver that plays a large role in metabolizing drugs. Many metabolic routes of elimination, including most of those occurring via the CYP450 family of enzymes, can be inhibited, activated, or induced by concomitant drug treatment. The FDA has recommended detailed studies be performed with the major CYP450 enzymes (CYP1A2, 2C9, 2C19, 2D6,

2E1, and 3A4). Typically, preclinical testing is performed to investigate the effects of an agent on metabolic factors, such as CYP450, and of inhibition or induction potential. If in vitro experiments reveal the potential for drug–drug interaction, in vivo experiments usually will follow. Therefore, phase 1 clinical trials often include testing for the ability of an experimental agent to affect CYP450 and a determination of whether the agent causes a change in concentration of other drugs as a result. With the combination of in vitro studies and in vivo studies in support of phase 1 clinical trials, the potential for drug–drug interactions can be studied early in the development process, with further study of observed interactions assessed later in the process, if necessary.

Organ Dysfunction Studies

The desirable and undesirable effects of a drug arising from its concentrations at the sites of action are usually related either to the amount administered (dose) or to the resulting blood concentrations (accumulation), which are affected by its absorption, distribution, metabolism and/or excretion. Elimination of a drug or its metabolites occurs either by metabolism, usually by the liver, or by excretion, usually by the kidneys and liver.

Although clinical trials for drug approval are often conducted in patients with normal hepatic and renal function, patients in clinical practice, especially those with cancer, may have compromised organ function because of underlying disease or from other causes such as aging, diabetes, infectious and autoimmune diseases, or drug-related toxicities (9). In general, drugs are approved and marketed with limited or no information on the pharmacokinetics and/or pharmacodynamics of the drugs in patients with organ dysfunction. Inadequate information in the drug label or in the scientific literature about the starting dose for organ dysfunction patients is of great concern to the treating physician who must manage the risk-benefit of these agents in patients with serious co-morbidities.

Phase 1 hepatic and renal dysfunction studies have been defined as clinical pharmacokinetic and pharmacodynamic experiments that represent prospective attempts to collect clinically useful dosing information from a difficult to study patient population and to formulate dosing recommendations based on these data (10). Phase 1 studies in organ dysfunction patients are typically carried out after a recommended dose has been established in an unimpaired patient population, and efficacy and safety have been established in phase 2 studies. It has been recommended that organ dysfunction studies be designed in the form of a formal dose-escalation phase 1 study, with a complete pharmacokinetic and toxicity profile as endpoints (9). The primary goal of the phase 1 study in an organ-impaired population should be to determine if the pharmacokinetics are altered to such an extent that the dosage requires adjustment, based on degree of organ dysfunction, from the dose established in the unimpaired population.

Due to the uniqueness of eligible patients, these studies are typically conducted as multisite studies to complete them in a timely and efficient fashion. In 1999, the National Cancer Institute (NCI) developed an Organ Dysfunction Working Group (ODWG), composed of approximately 12 to 15 phase 1 sites. The

ODWG has successfully completed evaluation of oxaliplatin and imatinib (STI-571) in the renal and hepatic impaired populations (11–15). In addition, several additional agents are currently undergoing evaluation.

Thorough QT Phase 1 Studies

Adverse effects on cardiac health have become one of the most common causes of product withdrawal from the market. As a result, regulatory authorities around the world have recently placed greater emphasis on cardiac safety. In May of 2005, the FDA endorsed the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) E14 document entitled: *The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs* (16). The FDA has indicated that new agents are expected to receive a clinical electrocardiographic evaluation, beginning early in clinical development, typically including a single, phase 1 trial designed to evaluate their effect on cardiac repolarization. This type of study has been referred to as a “thorough QT” study. A thorough QT study is described as a single trial dedicated to evaluating the effect a drug has on cardiac repolarization as a way to predict the risk of sudden death. According to the ICH E14 guideline, thorough QT phase 1 studies are to be performed to show that new investigational drugs do not change cardiac repolarization. The FDA’s regulatory guidance recommends a thorough QT phase 1 study to be conducted regardless of pre-clinical cardiac findings. When a thorough QT study is not feasible for other reasons, which may be the case in certain therapeutic areas such as oncology, alternative approaches are recommended, such as expanding the number and timings of electrocardiogram (ECG) recordings in other clinical studies in patients.

Dose-Scheduling Studies

Inefficient dose-scheduling can lead to treatment failure and the inadequate development of a potentially promising therapeutic. Unlike the typical phase 1 clinical trial designed to determine the MTD, an investigator may be interested in determining how often (i.e., how many administrations of a schedule) an agent could be safely administered to determine the long-term toxicity due to cumulative effects. Dose-scheduling studies are designed to determine the optimal administration schedule for an investigational agent.

Dose-scheduling studies can be combined with dose-finding studies or be completely separate studies. Pharmacokinetic and safety data obtained during a phase 1 dose-finding study may suggest it is feasible to increase the dose and/or reduce the frequency of administration of an agent, therefore indicating a dose-scheduling study is warranted.

Combination Studies

The typical phase 1 dose-finding study is designed to determine the MTD of a single, novel agent. However, an increasing number of patients, particularly in oncology, are being treated with drug combinations. The goal of a two-agent dose-finding trial is to find

the maximally tolerated dose of a dose combination (or combinations). Combination studies can be performed to determine the optimal dose and schedule of experimental drugs combined with standard chemotherapies and also of novel drugs combined together.

At the time that a combination study is designed, the monotherapy MTD doses of the individual agents under investigation are usually known. As a result, a minimal number of dose levels are typically needed to achieve the recommended dose of both drugs in combination. In the past, combination studies were routinely performed as single arm trials. However, recent novel designs, which include targeted and standard therapies, have been designed with several arms with differing standard therapies in combination with the novel agent under investigation (17). Such a design has been shown to expedite the identification of the combination MTD.

A common design for dose-finding studies with multiple agents is to investigate a single dose, or a small number of doses, of one agent and multiple doses of the second agent. If the study is combining a novel agent with a standard chemotherapy, the dose of the novel agent is usually varied while the standard chemotherapy is held to a single or a few doses.

Phase 1 Cancer Clinical Trial Designs

Phase 1 cancer clinical trials offer great excitement and hope for clinicians and patients alike as they often represent the end result of many years of preclinical work and new target identification. These trials are typically small, usually enrolling about 20 patients. However, this number can be quite variable depending on the trial design and the number of dose escalations, or cohorts, needed to determine the MTD. Many phase 1 clinical trial design methods have been proposed, and there is currently no consensus among the scientific, medical, and statistical communities on how best to perform these studies in humans.

Traditional Design

The most-commonly used design, often referred to as the traditional or “3 + 3” design, begins by assigning three patients in a cohort at a designated dose level, often one tenth the lethal dose (LD_{10}) in mice, scaled up to humans (18). Doses to be assigned are predefined by the investigators, based on preclinical data and clinical experience with similar agents, if it exists. One method of assigning successive dose levels uses a set of “increasing decreasing” Fibonacci dose level increments, usually 100%, 67%, 50%, 40%, and 33% for each dose level thereafter (19–21). These increments are added to each dose to get the next dose level. For example, the second dose level is 100% more than the first, the third dose level is 67% more than the second, the fourth dose level is 50% more than the third, and so on.

The decision whether to escalate to the next higher dose, expand a cohort, or de-escalate to a lower dose is made based on the toxicity information received from each three-patient cohort (Figure 47-1). If none of the three patients experience a

dose-limiting toxicity (DLT), the study proceeds to another cohort of three patients at a higher dose level (escalation phase). DLTs are predefined, and often include unacceptable drug-related toxicities as defined in grading scales commonly used in oncology, such as the NCI’s Common Terminology Criteria for Adverse Events (CTCAE). If one out of the three patients treated on a dose level cohort experience a DLT, up to an additional three patients (for a total of six) are treated at that dose level (expansion phase). If none of the additional patients experience a DLT, the dose will escalate. If at least two patients experience a DLT, the MTD is said to have been exceeded and the maximum administered dose (MAD) has been defined. An additional three patients will be tested at the next lowest dose level if there were only three patients previously treated at that level (de-escalation phase). In this particular case, the MTD is therefore defined as the highest dose level for which no more than one patient out of six experiences a DLT. Table 47-1 shows the different dose escalation/de-escalation decisions associated with toxicity outcomes at a given dose for an example of the 3+3 design.

Modifications to Traditional Design

A fundamental conflict in phase 1 trial design exists between escalating too quickly, resulting in the potential exposure of patients to excessive toxicity, and escalating too slowly, resulting in the treatment of patients at doses too low to be efficacious (19). A major criticism of the traditional phase 1 design is that the potential exists for many patients to be treated at subtherapeutic dose levels. In addition, the length of time these studies often take can inhibit the ability to rapidly bring new agents to subsequent phase 2 and phase 3 studies. Several variations to the traditional design have been developed to reduce the number of patients treated at doses below the biologically active level and to improve upon the precision of the MTD definition. Some of the most commonly used types of modified traditional designs include those proposed by Storer (22) and by Simon (18).

The Storer BD design uses a two-stage approach (22). In the first stage, only a single patient is entered at each dose level. Dose escalation continues with one-patient cohorts until a DLT is observed. Accrual to the second stage then begins at one lower dose level and follows the traditional (three-patient cohort) design. Such a scheme allows fewer patients to be treated at dose levels less likely to be efficacious. Storer also proposed defining the MTD by fitting the first-course toxicity data to a logistic dose-toxicity curve and letting the MTD be defined as the dose level associated with a target DLT rate (e.g., 20%–30%; 22). This allows for a more precise MTD definition.

Simon described three types of accelerated titration designs that were modifications of the traditional design (referred to by Simon as Design 1; 18). The Simon Design 2 is similar to the Storer design in that it uses single-patient cohorts during the initial stage, but the switch to the second stage (the traditional design) occurs when either the first instance of first-course DLT is observed or if two patients exhibit grade 2 toxicity, as defined by the CTCAE, during their first course of treatment (18). The Simon Design 3 mimics Design 2, except for the incorporation of

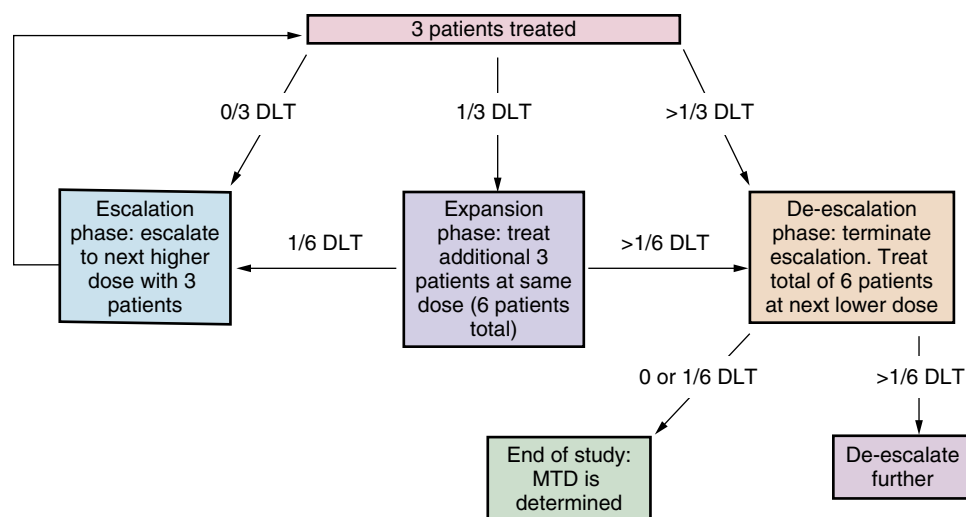


FIGURE 47-1 The traditional “3+3” phase 1 clinical trial design. The initial three-patient cohort begins at a predefined dose. If no dose-limiting toxicities (DLTs) are observed, escalation to the next higher dose will occur. If a single DLT is observed, expansion of the cohort to a total of six patients occurs. If more than one DLT is observed, de-escalation to the next lowest dose will occur for a total of six patients treated at that dose. Termination of the study will occur if more than one DLT is observed at the starting dose. The maximum tolerated dose (MTD) is defined as the highest dose level for which no more than one patient out of six experiences a DLT.

more rapid dose-escalation by using double-dose steps during the single patient cohort stage. Finally, the Simon Design 4 is similar to the Design 3, except switching to the second (three-patient cohort) stage may occur when either the first instance of a DLT occurs or the second instance of grade 2 toxicity is observed in *any* course of treatment. The three Simon accelerated titration designs also allow for inpatient dose escalation, permitting escalation for an individual patient if toxicity during their previous course was less than grade 2 as defined by the CTCAE and did not result in a DLT. Accelerated titration designs have become very popular, as they can dramatically reduce the number of patients required, shorten the duration of the trial, and provide a great deal of information about cumulative toxicity, interpatient variability, and steepness of the dose-toxicity curve (23). Most important, they provide all patients a maximum opportunity to be treated at a therapeutic dose. In reviewing several accelerated titration design phase 1 trials, it was identified that the advantages of its use from a prospective perspective were a minimal amount of patients needed to reach the MTD, a lower percentage of patients treated at potentially subtherapeutic doses or with an ineffective agent, and cost containment (24–28). However, accelerated titration designs did not expedite

the completion of studies overall, relative to traditional designs when compared with matching studies done in high-throughput phase 1 centers.

Cytotoxic versus Targeted Design

One of the assumptions inherent in the traditional phase 1 design is that both toxicity and clinical benefit will increase as the dose of an agent increases. For cytotoxic therapeutic agents, this assumption usually holds true. Recently, however, several agents have been developed that target specific tumor characteristics, such as receptors, and these agents may not follow the standard efficacy/toxicity model. Specifically, targeted agents may demonstrate a plateau on the dose-efficacy curve, meaning higher doses will not improve clinical benefit. In addition, toxicity occurring with the use of these agents, if it occurs at all, may not necessarily increase as the dose increases. For drugs of this type, determining the MTD may not be feasible or useful. For targeted agents that do not produce immediate or consistent drug-related toxicity, three categories of alternative endpoints have been considered: (1) measuring inhibition of a target, (2) plasma drug levels that are biologically relevant, and (3) surrogate markers of biologic activity in nontumoral tissues (29).

Several phase 1 trial designs have been developed for studies examining targeted, noncytotoxic agents (30–32). Hunsberger et al. (30) proposed several designs that are based on the assumption that there is a binary (positive or negative) response that is measured in each patient after treatment with an agent; this response indicates whether or not the desired effect has been achieved. The simplest of these designs mimics the traditional 3+3 design, but adapts it to examine response rather than toxicity. The goal of this design is to recommend the lowest dose meeting a predefined level of activity (response) for further testing. Dose escalation occurs when a predefined number of responses are not observed. Dose de-escalation will occur if the predefined level of responses has been exceeded.

Pharmacokinetically Guided Dose-Escalation Method

The pharmacokinetically guided dose-escalation (PGDE) method of clinical trial design was proposed by Collins et al. as a more

Table 47-1 Dose Escalation/De-escalation Decisions Associated with Toxicity Outcomes at Given Dose For Popular Version of 3+3 Design

No. Patients with Dose-Limiting Toxicity	Decision
0/3	Escalate one level
1/3	Treat 3 more at same level
1/3 + 0/3*	Escalate one level
1/3 + 1/3*	Stop and chose previous dose as the MTD
1/3 + (2/3 or 3/3)*	Stop and chose previous dose as the MTD
2/3 or 3/3	Stop and chose previous dose as the MTD

* Note that those rows with number of toxicities equal to $1/3 + t/3$ (for $t=0, \dots, 3$) corresponds to situations in which one toxicity is observed in the first cohort of three patients enrolled at the current dose and t toxicities are observed in the second cohort of patients enrolled at that dose.

informative and efficient alternative to the traditional design (19,33). The authors retrospectively analyzed the results of several phase 1 studies of chemotherapeutic agents, and demonstrated that observed toxicity was not a function of the dose administered to the patient, but rather was a function of the area under the curve (AUC) of plasma drug concentration measured over time of exposure. The PGDE phase 1 clinical trial design targets the AUC associated with the mouse LD_{10} . Patients are treated at one tenth of the mouse LD_{10} , as in the traditional method, but escalation to the next dose and subsequent doses is based on the distance of the observed AUC in humans to the target mouse LD_{10} AUC. The retrospective analysis performed by Collins et al. indicated that the sample size of phase 1 clinical trials could be reduced by up to as much as 50% by using the PGDE over the traditional design (19). Although several studies have reported success using the PGDE design, it is still not widely used in the drug development community (34). One reason for the lack of use is the presence of large interpatient variability in AUC for the same administered dose (23). For some drugs (e.g., antineoplastic and vinca alkaloids), toxicity is a function of exposure time rather than AUC, and the use of a PGDE design is not justified (35). Finally, the requirement of real-time pharmacokinetic monitoring inherent in the PGDE design has been considered a limitation to its use (34,36). Pharmacokinetic correlative studies, however, have become standard measurements in almost all oncology phase 1 trials as they help to better understand phase 1 trial outcomes.

Continual Reassessment Method

O'Quigley et al. (37) proposed the continual reassessment method (CRM) as an alternative phase 1 study design. This phase 1 design utilizes formal statistical methods of dose-toxicity modeling to guide dose escalation. The CRM is considered superior by many because it allows the use of toxicity information gained at earlier time points of the study to assign subsequent doses. The CRM design is considered less likely to treat patients at toxic doses, and more likely to treat patients at doses considered efficacious (38). The CRM, as originally designed, works by fitting a dose-toxicity curve to the available toxicity data and assigns subsequent patients to the dose most likely to be associated with a predefined target toxicity level. Therefore, the MTD is defined as the dose estimated to produce a desired predefined toxicity rate. The estimated dose-toxicity curve is refit after the outcome of each individual patient is determined, and the next patient is assigned the dose estimated to be nearest the MTD based on the new data (38). Due to its complexity, involvement of a capable statistician is necessary in the design and execution of a CRM-designed clinical trial.

Statistical Considerations of Phase 1 Studies

There are many designs available to estimate the MTD, as discussed previously. Two of the main design types used in practice are either algorithmic in nature (e.g., the previously described 3+3 design) or model-based designs (i.e., designs based on a statistical model). The purpose of the 3+3 design is not to produce accurate estimates of the probability of toxicity at a given dose but to quickly

identify a dose level that does not exhibit too much toxicity. An alternative to algorithmic approaches such as the 3+3 design and one more amenable to the goal of precisely estimating (i.e., estimating with more certainty) the MTD are model-based methods. The conceptual frameworks for most model-based phase 1 designs are Bayesian in nature. Bayesian designs treat the probability that a patient will experience toxicity at a given dose as a quantity about which the investigator has some degree of uncertainty. Moreover, this uncertainty is quantified via probability. The Bayesian framework provides a means by which one can learn about the toxicity rates at the different doses, and naturally make decisions based on the data observed in a sequential manner.

Using these model-based designs requires that the investigator *explicitly* specify a target probability of toxicity. The target probability of toxicity represents the rate of toxicity acceptable to the investigator. (The 3+3 design has an *implicit* target rate of toxicity of approximately 17%.) For compounds associated with very severe life-threatening toxicities, the target probability may be set by the investigator at 0.10 (i.e., 10%), whereas for other compounds with more mild toxicities it may be acceptable to set the target probability of toxicity at 0.35. As with algorithmic designs, patients are sequentially enrolled into the trial in cohorts of patients. After each cohort of patients has been evaluated for toxicity, the decision to escalate, stay, or de-escalate from the current dose is based on the dose that has the expected probability of toxicity closest to the target toxicity.

An important advantage of model-based phase 1 designs is that they allow one to combine information from patients treated at different dose levels, that is, to “borrow strength,” to more reliably predict what may occur at a particular dose given to a future patient. A second advantage is the ability to adjust the target probability of toxicity to match the characteristics of the compound under investigation. A third advantage of model-based methods is, unlike the 3+3 design, that the cohort size is not limited to three patients, and more important, a variable cohort size may be used. Although one could argue that algorithmic designs can also use alternative cohort sizes, the complication associated with changing the cohort size when using “X+X” algorithmic approaches (i.e., 2+2, 4+4, 5+5, etc.) is that the implicit targeted rate toxicity changes with the size of the cohort. We should note that there are other algorithmic designs which do not tie the implicit target toxicity rate to the cohort size but these methods are very rarely used and tend to place too many patients on doses which are too toxic (reviewed [39]).

Although model-based designs have been available since the early 1990s, these methods have not gained as wide an audience as biostatisticians would like. This is because it can be difficult to explain these methods to nonstatisticians and difficult to implement (40). These difficulties are being addressed by making computer code available to investigators and by providing innovative designs which target endpoints other than the typical endpoint in a classical implementation of a phase 1 oncology design. Some of these innovative designs are discussed in the following sections.

One interesting innovation is modeling time to toxicity as opposed to toxicity as a binary outcome. Using this strategy, each cohort of patients does not have to be completely followed before

the next patient or group is assigned a dose (unlike traditional designs for phase 1 clinical trials). This design has most appeal in cases where one wishes to evaluate the safety of a new compound over a long period of time (i.e., 3 months or longer). Using a traditional approach would result in trials that are excessively long. A new method, called the time-to-event continual reassessment method (41), allows patients to be entered into a trial before all patients currently enrolled have been completely observed. Using this method, a trial enrolling 24 patients in cohorts of size 3 and utilizing a toxicity assessment window of 90 days, which would take 3 years to complete using a traditional method, could be reduced to 15 months using the time-to-event method.

Another innovation includes phase 1 trials focused on schedule finding rather than dose finding. A new method based on determining the maximum-tolerated schedule (MTS) rather than a conventional MTD addresses this type of trial design (42). The method accounts for a patient's entire sequence of administrations, with the overall hazard of toxicity modeled as the sum of a sequence of hazards, each associated with one administration.

New developments that have received much research interest are adaptive Bayesian methods for dose-finding in phase 1/2 clinical trials based on tradeoffs between the probabilities of treatment efficacy and toxicity. Studies include O'Quigley, Hughes, and Fenton (2001); Ivanova (2003); Braun (2002); and Thall and Cook (2004; 43–46). These methods are most effective when toxicity and efficacy are not correlated through dose or only correlated through dose for a subset of doses. These trials are fundamentally different from the typical phase 1 oncology trial where toxicity is assumed to be correlated with efficacy through dose.

An interesting advance in phase 1/2 dose finding includes the work by Bekele and Shen (2005) in which they propose a new Bayesian approach to dose-finding in oncology trials by jointly modeling a binary toxicity outcome and a continuous biomarker expression outcome (47). They applied their method to a clinical trial of a new gene therapy for bladder cancer patients. In this trial, the biomarker expression indicates biologic activity of the new therapy. For ethical reasons, the trial is conducted sequentially, with the dose for each successive patient chosen using both toxicity and activity data from patients previously treated in the trial. The modeling framework they use naturally incorporates correlation between the binary toxicity and continuous activity outcome via a latent Gaussian variable. They show that the design reliably chooses the preferred dose using both toxicity and expression outcomes under various clinical scenarios.

The work by Bekele and Thall, in which toxicity is modeled as a set of ordinal toxicity outcomes, was successfully used to model the relationship between toxicity and dose in a phase 1 trial of gemcitabine for soft-tissue sarcoma (48). This approach was taken because, in phase 1 oncology trials of new cytotoxic agents, patients typically are at risk of several qualitatively different toxicities, each occurring at several possible severity levels. The oncologists planning the trial desired to account for differences in importance among several toxicities, and they wanted the dose-finding method to use the information that a low-grade toxicity observed at a given dose is a warning that a higher grade of that toxicity is likely to occur at a higher dose. Because conventional

methods do not address these issues, they developed a Bayesian method for dose-finding based on a set of correlated, ordinal-valued toxicities with severity levels that vary with dose. They also developed a method for eliciting the set of weights quantifying the clinical importance of each level of each type of toxicity, and the physicians' target total toxicity burden.

Pharmacodynamic Markers in Phase I Studies: Tissue Analysis

Overview of Pharmacodynamic Markers in Tissues

In recent years, there has been significant progress in the development of drug-targeted therapies, particularly those that target receptor tyrosine kinases (RTKs; 49,50). The rapid emergence of hundreds of molecular agents against numerous targets offer greater anticancer efficacy with fewer side effects. Despite these recent advances, assessing the effects of these agents individually or in combination, or combined with conventional therapies, has created significant challenges for basic scientists and clinical investigators to effectively integrate molecular targeted therapies into clinical practice (51). Because the number of possible drug–target combinations is limitless, better strategies are needed to understand the pharmacodynamic effects of investigational agents in tumors (52). One of the most informative approaches is to implement correlative tissue-based analyses in clinical studies (53). Although tumor biopsies are not accessible in every cancer type, data obtained from correlative studies may help determine (1) whether an agent hit its intended target, (2) whether the target inhibition is transient or stable to induce apoptosis in specific cell types (e.g., in endothelial or tumor cells), (3) optimization of dosing or scheduling, (4) biomarkers that indicate which patients are most likely or least likely to respond, and (5) mechanisms of actions and resistance of single agents and their combinations. This section discusses the development of reliable assays for quantifying pharmacodynamic effects in tissues, the effects of different agents on various markers and their correlation with clinical outcome, and issues that pose challenges for incorporating tumor tissue analysis into clinical trials.

Quantitative Analysis of Pharmacodynamic Markers in Tissues

Investigators typically rely on immunohistochemistry assays to measure the pharmacodynamic effects of molecular targeted therapies in tissues. Most studies use chromogenic or immunoperoxidase staining, which are semi-quantitative in nature and have other limitations (54). In contrast, immunofluorescence detection methods can provide simultaneous labeling of multiple proteins in one sample and a quantitative assessment using a continuous scale (55). Recent research efforts have focused on the development of immunofluorescence-based assays to quantify protein expression patterns and apoptosis in tissues for phase 1 studies (Figure 47-2; 56,57). Initially, this work focused on developing a method to detect apoptosis in endothelial cells, which requires

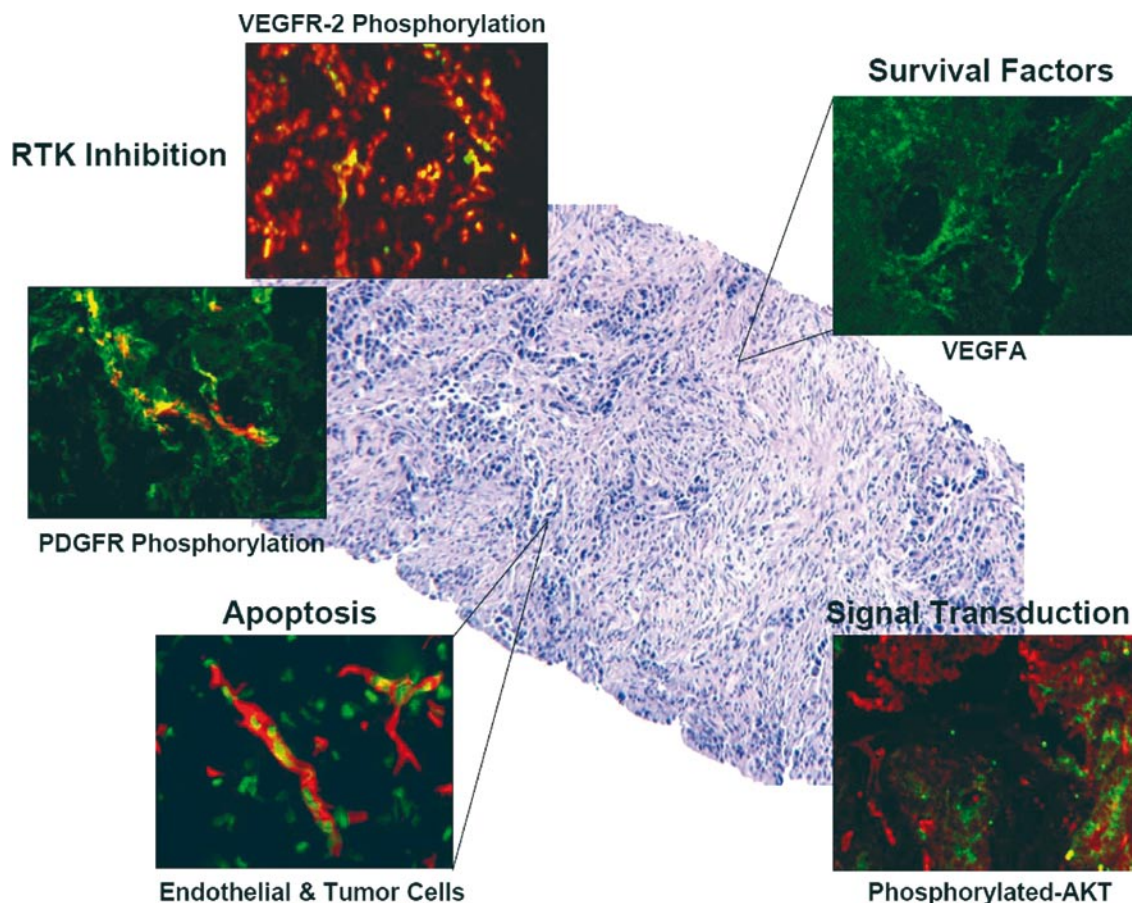


FIGURE 47-2 PHARMACODYNAMIC ANALYSIS OF MOLECULAR TARGETED THERAPIES IN TUMOR TISSUES. Correlative tissue studies may help determine the pharmacodynamic effects of targeted therapies on receptor tyrosine kinase phosphorylation, growth factors, signal transduction, and apoptosis in phase 1 studies. Immunofluorescence detection permits the analysis of biomarkers in specific cell types (e.g., phosphorylation of platelet-derived growth factor receptor- β [PDGFR- β]) in endothelial cells. Measuring endpoints that include target or pathway inhibition linked to apoptosis may provide better evidence of the biologic effects of the drug in the tumor and correlation with clinical outcome. Red, endothelium; green, protein expression or terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL); yellow, colocalization of endothelium and protein or TUNEL.

three fluorochromes to visualize the total cell nuclei, endothelial cells, and terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL)-positive cells (58). Hence, multiple labeling techniques can facilitate visualization of specific cell types by eye as a result of colocalization of different fluorochromes (Figure 47-2). However, manual quantification is limited to enumerating “positive” and “negative” cells in random microscopic fields using a categorical score and may not be able to detect subtle, but significant changes (59).

Various platform technologies have been developed to facilitate quantitative in situ assessment of protein expression (60). Most of these systems are designed for standard immunohistochemistry assays using chromogenic substrates. Measuring the pharmacodynamic effects of molecular targeted therapies requires the ability to detect specific cell types (e.g., endothelial cells) and quantify their protein expression patterns. One platform technology capable of quantifying multiple fluorochromes in fixed tissue specimens is the laser scanning cytometer (LSC). The LSC platform is an automated analysis system described as a cross between a flow and a static image cytometer. Lasers are used to simultaneously excite different fluorochromes in cellular specimens that emit discrete wavelengths detected by a set of photomultiplier tubes.

Together, these features permit the ability to generate high-content stoichiometric data on heterogeneous populations of large numbers of cells. Thus, the LSC is used much like a flow cytometer to obtain multicolor immunofluorescence intensity information on fixed specimens.

Several phase 1 studies have incorporated LSC-mediated analysis to determine drug-target interactions, effects on downstream signaling pathways, and rates of apoptosis in skin and tumor tissues (55–57,61). Because the LSC is a platform technology, many different applications can be developed to exploit its inherent capabilities. Research efforts have been focused on developing specific tissue-based applications using LSC technology in an attempt to standardize the methodology for consistent data generation that can be compared between different tissue specimens and molecular targeted therapies. Although LSC-mediated data acquisition is automated, the process requires a systematic interactive approach to maintain high quality control standards and ensure consistent data generation (Figure 47-3). Pharmacodynamic data generated using a process to analyze markers in entire tumor tissue cross sections has consistently provided biologic evidence of the effects of targeted therapies and correlation with clinical outcome (55,62,63). A summary of phase 1 studies of targeted therapies

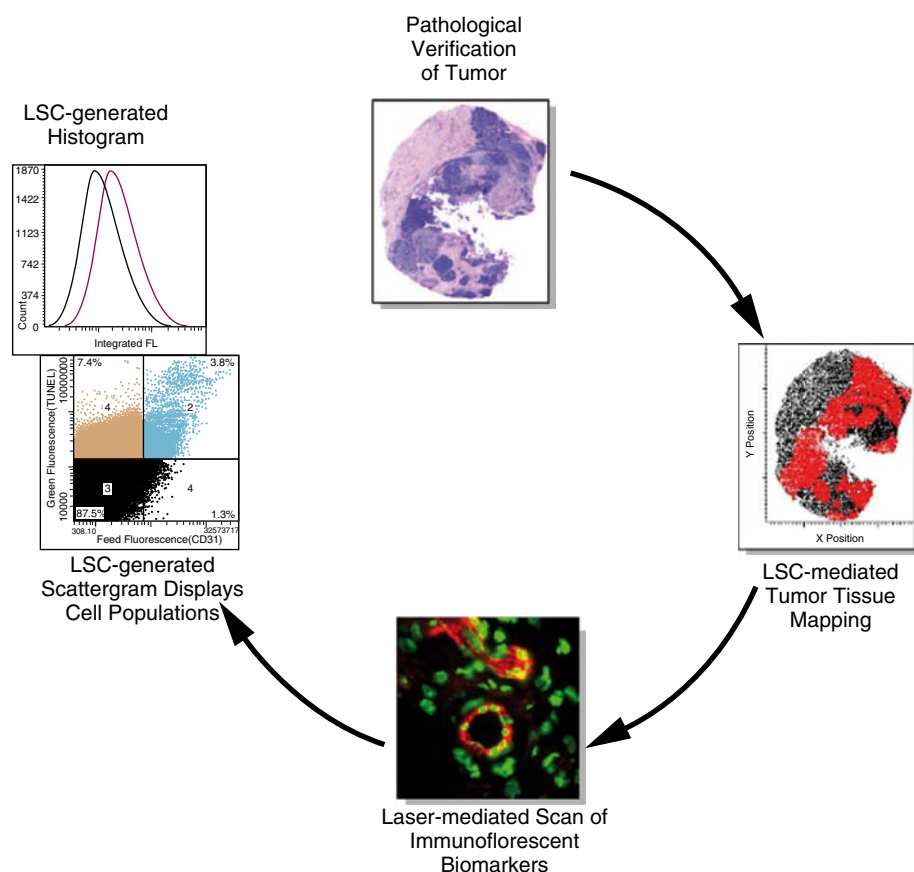


FIGURE 47-3 Quantitative analysis of pharmacodynamic effects in tissues using laser scanning cytometer (LSC) technology. Pathologic verification of biopsy samples is essential for mapping tumor regions and excluding normal and necrotic regions from the analysis. Lasers detect individual cells within the mapped region of interest based on immunofluorescence staining. LSC-generated scattergrams display the percentage of cell populations based on user defined gating using controls (e.g., apoptotic endothelial cells). Alternatively, protein expression levels (e.g., phosphorylated VEGF receptor-2) measured by mean fluorescent intensity may be determined as shown in the histogram. (Immunofluorescent image (c) 2000 BioTechniques, used by permission.)

that have incorporated pharmacodynamic analysis of tissues, their effects on various pathways and correlation with clinical outcome are shown in [Table 47-2](#).

Pharmacodynamic Analysis of Receptor Tyrosine Kinase Targeted Therapies

Aberrant expression of cell-surface RTKs (e.g., epidermal growth factor receptor [EGFR]) plays a pivotal role in the progression of cancer (64). Drugs that target RTKs are designed to block the intrinsic enzymatic activity that catalyzes the transfer of the gamma-phosphate of ATP to tyrosine residues in protein substrates (65). Inhibiting phosphorylation of these tyrosine residues prevents downstream signaling events, which affect cellular function (e.g., proliferation, differentiation, migration or apoptosis; 66). Thus, the ability to measure phosphorylation status and signal transduction pathways has become an important pharmacodynamic endpoint in clinical studies.

ZD1839

ZD1839 (Iressa, Gefitinib) was the first in a new class of small, molecular-targeted therapies against EGFR to gain market approval (based on two phase 2 studies) for non-small cell lung cancer (NSCLC; 67,68). Although the phase 2 studies did not incorporate correlative tissue studies, two different phase 1 studies of ZD1839 demonstrated that pharmacodynamic endpoints

can be measured in both tumor and skin tissues. In a metastatic colorectal cancer trial, total EGFR was detected by immunohistochemistry in all 16 pretreatment tumor biopsies (69). Total EGFR levels remained unchanged in seven of ten patients after treatment with ZD1839 whereas the other three demonstrated a decrease. Interestingly, two of the patients whose EGFR levels decreased displayed a large increase in apoptosis. Phosphorylation of EGFR was also measured; however, it was reported that detection was reproducible in only one patient and that this patient displayed complete inhibition after treatment with ZD1839. Other downstream markers of the EGFR signaling pathway were measured including phosphorylation of AKT and extracellular receptor kinase (ERK), p27^{Kip1}, and β -catenin expression. Phosphorylated-AKT and ERK (in tumor cells) were decreased in two and one patients, respectively. However, phosphorylated-ERK in tumor stromal fibroblasts was lower in five of nine patients after ZD1839 therapy. Daneshmand and coworkers suggested that it is likely other patients had activated EGFR and AKT but at levels that were below the detection limit of standard immunohistochemistry. The cyclin-dependent protein kinase p27^{Kip1} was not detected in seven of nine patients, in part because EGFR activation promotes degradation of p27^{Kip1} (70). After ZD1839 therapy, levels of p27^{Kip1} increased in two patients, whose tumors also displayed an increase in apoptosis. ZD1839 treatment did not affect β -catenin expression in three of the paired samples that were evaluated. The proliferation index, measured by Ki67, was the only marker in this study that significantly correlated with change in tumor burden. Interestingly, the two patients who

Table 47-2 Analysis of Pharmacodynamic Markers in Tissues from Phase 1 Clinical Studies

Agent ^{Reference}	Target	PD Markers	Change in Expression on Therapy	Correlation	Response
ZD1839 ⁶⁹ (Iressa, Gefitinib) Metastatic Colorectal Cancer	EGFR TKI	EGFR pEGFR pAKT pERK p27 Beta-Catenin TUNEL Ki67	7 of 10 No Change, 3 Decrease 1 of 16 Decrease 2 of 10 Decrease 5 of 9 Decrease in Tumor Fibroblasts 2 of 9 Increase in Nucleus 2 of 9 No Change 6 of 10 Increase, 2 No Change, 2 Decrease 8 of 10 Decrease*	None None None None None None None None With tumor burden	None
ZD1839 ⁷¹ (Iressa, Gefitinib) Metastatic Breast Cancer	EGFR TKI	<u>Tumor</u> ERBB2 EGFR	No Change No Change	None None	2 PR, 7 SD
ZD1839 ⁷² (Iressa, Gefitinib) Advanced Solid Tumors	EGFR TKI	<u>Skin</u> EGFR pEGFR pMAPK pSTAT3 p27 TUNEL Ki67	No Change Decrease* Decrease* Increase* Increase* Increase* Increase* Decrease*	None Compared to Pretreatment Compared to Pretreatment Compared to Pretreatment Compared to Pretreatment Compared to Pretreatment Compared to Pretreatment	7 SD
EMD 72000 ¹³² Advanced Solid Tumors	EGFR mAB	<u>Skin</u> EGFR pEGFR pMAPK pSTAT3 TGF-alpha p27 Ki67	No Change Decrease* Decrease* Increase* No Change Increase* Decrease*	None Compared to Pretreatment Compared to Pretreatment Compared to Pretreatment None Compared to Pretreatment Compared to Pretreatment	5 PR, 6 SD, 1 MR
SU5416 ¹³³ Advanced Solid Tumors	VEGFR-2 TKI	<u>Tumor</u> MVD	5 or 19 Increase	None	4 SD
SU6668 ^{55, 61} Advanced Solid Tumors	VEGFR-2 and PDGFR TKI	<u>Tumor</u> pVEGFR-2 EC & TC pPDGFR EC TUNEL TC TUNEL MVD	1 of 6 Decrease 1 of 6 TC Decrease No Change No Change No Change	Transient target inhibition Transient target inhibition Transient target inhibition Transient target inhibition Transient target inhibition	None
Endostatin ¹³⁴ Advanced Solid Tumors	Endogenous angiogenesis inhibitor	<u>Tumor</u> MVD CD31 + Ki67 vWF + TUNEL <u>Skin</u> MVD CD31 + Ki67 vWF + TUNEL	No Change No Change Rare Event No Change Slight Decrease Rare Event	None None None None None None	SD
Endostatin ^{56, 75} Advanced Solid Tumors	Endogenous angiogenesis inhibitor	<u>Tumor</u> MVD CD31 + TUNEL BCL-2 HIF-1 TC + TUNEL	Decrease* Increase* No Change No Change No Change	With optimal dose & response With optimal dose & response None None None	1PR, 1SD
CI-1040 ⁷⁸ Advanced Solid Tumors	MAPK 1 & 2	<u>Tumor</u> pERK	Decrease	Target inhibition, median 73%	1 PR, 19 SD

Table 47-2 Analysis of Pharmacodynamic Markers in Tissues from Phase 1 Clinical Studies—Continued

Agent ^{Reference}	Target	PD Markers	Change in Expression on Therapy	Correlation	Response
PS-341 ⁸² (Bortezomib) Advanced Solid Tumors	PS-341 Reversible Proteasome Inhibitor	<u>Tumor</u> p27	1 27-fold Increase	Confirmed Therapeutic Response	1 PR, 1 SD
BMS-214662 ⁸⁶ Advanced Solid Tumors	Farnesyltransferase Inhibitor of H-ras K-ras	<u>Tumor</u> Total & p-MAPK Total & p-AKT p27 Ki67 TUNEL Caspase 3 & 9	None None None None Increase* Increase*	None None None None With Dose and Schedule With Dose and Schedule	5 SD
17-AAG ¹³⁵ Advanced Solid Tumors	HSP90 - ATP Inhibitor	<u>Tumor</u> HSP70 CDK4 RAF-1	Increase Decrease Decrease	Identified optimal schedule	2SD
Ad5CMV-p53 ¹³⁶ Advanced Solid Tumors	Adenoviral Vector Containing Wild-Type p53 Gene	<u>Tumor</u> p53 <u>Skin</u> p53	6 of 7 Increase 1 of 4 Increase	Confirmed delivery Confirmed delivery	1SD

MR, major response; PR, partial response; SD, stable disease; TKI, tyrosine kinase inhibitor.

* Significant change.

had the largest decrease in Ki67 also showed the largest increase in apoptosis. In another phase 1 study of ZD1839 in metastatic breast cancer, comparison of pre- and post-treatment ERBB2 and EGFR values was not statistically significant between the subgroups of patients regarding responsiveness to treatment (71).

Serial skin biopsies have been analyzed as potential surrogate tissues for monitoring the biologic effects of molecular targeted therapies. A phase 1 study of ZD1839 in advanced solid malignancies incorporated skin, but not tumor, biopsies to determine effects on EGFR signaling (72). Levels of phosphorylated-EGFR expression were completely inhibited; however, no changes in total EGFR expression were observed after treatment. Other downstream markers in the EGFR network were affected by ZD1839 including phosphorylated-Ras-mitogen-activated protein kinase (MAPK) and STAT3, Ki67, p27^{Kip1}, and apoptosis (Table 47-2). Although significant changes were observed in almost all of the markers when comparing pre- and post-treatment skin biopsies, none of the changes correlated with dose or clinical response.

Pharmacodynamic Analysis of Signal Transduction Inhibitors and Other Targets

Endostatin

Endostatin is a COOH-terminal cleavage fragment of collagen XVIII that can function as an endogenous inhibitor of tumor angiogenesis (73,74). Endostatin's receptor(s) has not been isolated, and its mechanism of action remains obscure. Low levels of apoptosis were observed in a phase 1 dose-finding study (15–600 mg/m²) of endostatin, and it was apparent that more sensitive quantitative methods were required (57,75). Using the LSC-mediated quantitative analysis technique, it was demonstrated that the average level of apoptosis significantly

increased from 0.2% to 1.1% after endostatin therapy (56). Although the levels of apoptosis were low, a fivefold increase is comparable to tumor cell lines exposed to cytotoxic agents in vitro. In addition, significant decreases in microvessel density were observed following endostatin treatment. Effects on the vasculature were quantified using an innovative technique to enumerate microvessels in an entire tissue cross-section. Other parameters quantified by LSC that did not show significant changes with dose were BCL-2 and hypoxia-inducible factor-1 α . Intriguingly, the changes in endothelial cell apoptosis and microvessel density were consistent with significant decreases in blood flow measured by positron emission tomography. Together, these data strongly suggested that endostatin had optimal biologic activity at doses of 250 mg/m² in this cohort of patients (56).

CI-1040

MAPK plays a key role in mediating growth-promoting signals from multiple growth factor receptors. Specifically, MAPK catalyzes the phosphorylation of its substrates, ERK-1 and ERK-2 (76). CI-1040 is a highly potent and selective inhibitor of both MAPK isoforms as evidenced by an IC₅₀ (concentration associated with 50% inhibition of MEK) of 17 nmol/L against purified MEK1 (77). In a phase 1 study of CI-1040 (78), reduced levels of phosphorylated-ERK in tumor tissue of 50% were used as part of the decision to continue clinical development of the compound. This development objective was achieved based on the 73% median decrease in phosphorylated-ERK observed in ten patients.

PS-341

The 26S proteasome is a multicatalytic protease that selectively degrades polyubiquitinated proteins, primarily short-lived intracellular

proteins that regulate cell cycle, tumor growth, and survival (79,80). Inhibition of the 26S proteasome results in the disruption of cell cycle checkpoints and apoptosis pathways. PS-341 is a potent and specific (K_i 0.6 nmol/L) reversible proteasome inhibitor that was rapidly approved (4.5 years) for the treatment of multiple myeloma (81) and is under clinical development for solid tumors. A phase 1 study was conducted to evaluate pharmacodynamic endpoints comparing two different schedules (82). Unfortunately, tumor tissue was limited and only one evaluable patient demonstrated a 27-fold increase in p27^{Kip1} expression after PS-341 treatment. These data combined with other pharmacodynamic data obtained from peripheral blood mononuclear cells correlated with 70% inhibition in proteasome activity and provided evidence of PS-341-induced biologic effects in the tumor.

BMS-214662

Ras proteins play a key role in a large number of cellular processes including growth, differentiation, apoptosis, membrane trafficking, and cytoskeletal organization (83,84). After synthesis, these proteins are post-transcriptionally modified by the farnesyltransferase enzyme to a more hydrophobic state that permits its localization to the cytoplasmic membrane to mediate RTK signaling. BMS-214662 is a small-molecule inhibitor of human farnesyltransferase of both H-ras and K-ras, with an IC_{50} of 1.3 and 8.4 nM and an IC_{90} of 18 and 108 nM, respectively (85). Tabernero and coworkers assessed the effects of BMS-214662 on signal transduction proteins hypothesized to play a key role in signaling in tumor tissues (86). Surprisingly, BMS-214662 did not induce changes in total MAPK or AKT, phosphorylation of MAPK and AKT, p27^{Kip1}, or Ki67. In contrast, a strong increase in tumor cell apoptosis was observed as evident from an increased expression of cleaved caspase 3 and 9, and TUNEL. Together, these data suggest that BMS-214662 acts through a Ras-independent pathway similar to other farnesyltransferase enzymes (87,88). Although the levels of apoptosis correlated with schedule and dose, there was no direct correlation with clinical benefit. These data are consistent with the fact that no objective tumor responses were observed.

Challenges and Perspectives

There are many challenges to successfully incorporating tissue analysis in the design of a clinical study. Acquiring the tissue alone requires the commitment of the sponsor, scientists, oncologists, interventional radiologists, committees, and patients. Standardization of tumor sampling and tissue procurement is critical to ensure that quality tumor tissue is being evaluated. A lack of quantitative standardization among different assays may lead to unintentional interpretation and variability between laboratories. Other issues that may impact interpretation of pharmacodynamic data are intra- and intertumor heterogeneity, tissue microenvironment (skin vs. tumor), compensatory mechanisms, and timing of biopsies after initiation of therapy and after the last dose. It is worth emphasizing that few studies have attempted to link target or pathway inhibition with tumor cell apoptosis. It is possible that some agents may demonstrate transient target inhibition, but fail to induce apoptosis (55). Thus, measuring pharmacodynamic endpoints that include target or pathway inhibition linked to cellular fate (e.g., apoptosis) may provide better evidence of the biologic effects of the drug in the tumor.

Pharmacodynamic analysis of tumor tissues can provide direct proof of whether an investigational agent affected its intended target and downstream consequences on signal transduction and apoptosis, however, they are also limited. Recent studies have demonstrated that skin may serve as a surrogate tissue to confirm drug-target inhibition, signal transduction, and kinetics in clinical studies. However, analysis of biomarkers in tumor tissues may better represent the biologic effects of a targeted therapy as tumor cells often respond differently compared to normal cells. More quantitative studies are needed to identify reliable biomarkers and their correlation between the effects in skin, tumor, and clinical outcome. Another promising surrogate source that could potentially be used to assess the effects of targeted agents is the circulating tumor cell or endothelial cell. These cells may better represent the tumor microenvironment and are now being routinely isolated for a variety of applications (88). Our ongoing research efforts are aimed at developing assays to analyze the pharmacodynamic effects of circulating tumor and endothelial cells. Furthermore, pharmacodynamic studies in tumor tissue may also identify the genomic and proteomic profile of the population with the greatest chance to benefit from treatment. For example, the therapeutic activity of Herceptin would likely have been missed if patients had not been pre-selected based on their HER2 status.

Clearly, there is a need for better strategies to assess the effects of molecular targeted therapies early in clinical development. For example, in a phase 1 trial of bevacizumab, no objective responses were observed out of 25 patients (89). It was not until a series of randomized phase 2 and 3 trials over a period of more than 5 years that the clinical activity of bevacizumab was established. However, it is generally not practical to perform large randomized trials for drugs without evidence of biologic activity early in their development, and therefore, many promising drugs may not be developed. Given the large number of targeted therapies entering clinical testing, it is crucial that phase 1 studies incorporate correlative endpoints to determine optimal dosing and scheduling for phase 2 and 3 trials. Ultimately, clinical development of targeted therapies would benefit if the recommended dose was identified early and actually known to inhibit the target for which it was designed.

Imaging Techniques in Phase 1 Studies

A variety of imaging techniques can play an important role in phase 1 studies of anticancer drugs when used as an objectively measured indicator of a biologic/pathobiologic process or pharmacologic response to treatment (i.e., as a biomarker [90]). Imaging biomarkers can be used to determine if the drug is hitting the target, if it has the anticipated biologic activity, and can also provide an early indication of whether or not the new agent has clinical activity (91). The information provided by imaging biomarkers, taken together with information from molecular biomarkers and clinical pharmacology, provide the input required to determine how aggressively to pursue development of a particular drug or a backup drug for a given target. For example, if there is no evidence for the anticipated biologic activity of a drug candidate, evidence that the drug did not hit the target (or only at an insufficient level) would support

development of a back-up drug. However, if the candidate drug does hit the target, it would not make sense to pursue development of a backup drug. In addition, imaging biomarkers can assist in the selection of the dose and/or schedule for phase 2 studies (92).

The ability to detect labeled drug at thousand-fold lower concentrations than needed to produce pharmacodynamic effects makes nuclear medicine the best suited modality for determining if the drug is hitting the target (93). Small molecules can be labeled with the positron emitting nuclides ^{11}C or ^{18}F and larger molecules (e.g., proteins or antibodies) can be labeled with the positron emitting nuclides ^{124}I or ^{64}Cu (94–97). Alternately, other specific receptor ligands can be labeled with radionuclides and used to measure receptor occupancy (98). Although none of these methods have been used to a great extent in phase 1 oncology studies to date, they could provide a powerful approach for obtaining information about how much drug reaches the target. It should be noted that the Pharmacodynamic/Pharmacokinetic Technologies Advisory Committee of Cancer Research United Kingdom recently argued that measuring downstream biologic effects are likely to be more cost-effective than those that measure specific molecular targets (99). This concern may be most relevant for small therapeutic molecules, since each molecule may require unique labeling methods and some may not be amenable to radiolabeling. However, the methods used for labeling biomolecules are more general, so may be more readily applied to a variety of drugs. This is of particular importance given the greater concern regarding delivery of macromolecules to solid tumors (100).

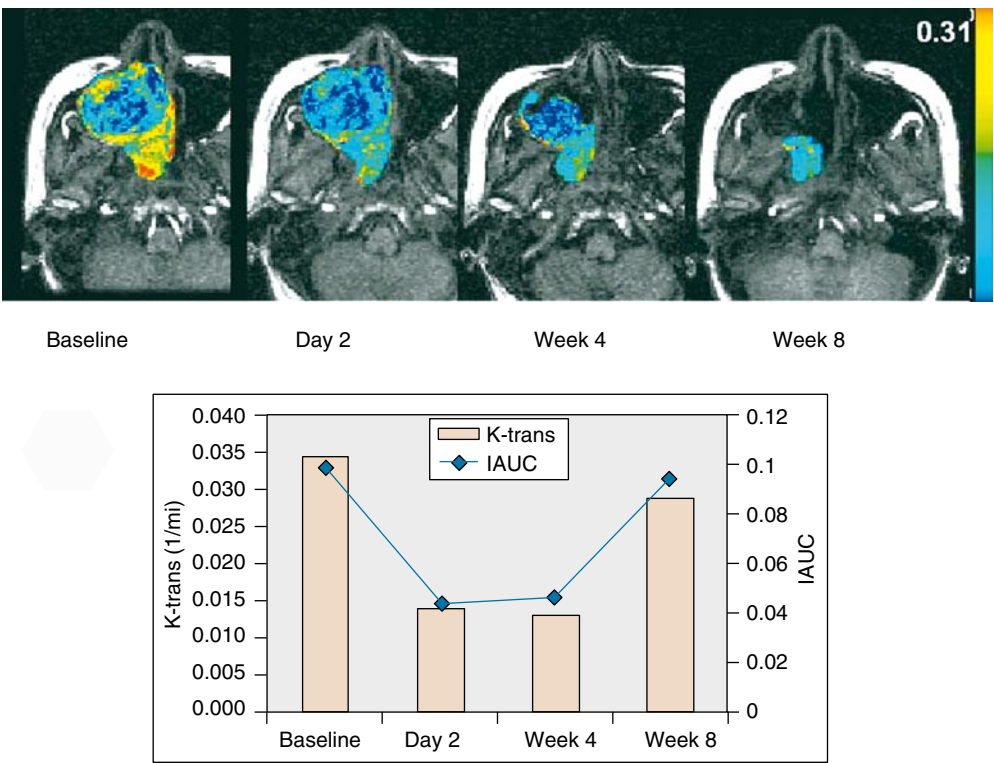
A number of imaging modalities can be used to determine if the drug has the anticipated biologic activity. The more commonly used methods are dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and [^{18}F] fluoro-2-deoxyglucose (FDG) positron emission tomography (PET). Other promising imaging biomarkers under development for oncology include ^{11}C -thymidine, FLT or FMAU for proliferation (101), Annexin-V or specific caspase-3 activity tracers for apoptosis (102,103), diffusion MRI for cellularity (104,105), and labeled RGD (106), $\alpha_v\beta_3$ (107,108), or tracers for other specific vascular targets (109). These pharmacodynamic biomarkers promise to provide a valuable set of measures of downstream biologic effects for nearly any molecular target.

DCE-MRI, which uses a commonly used contrast agent (gadopentetate dimeglumine), has been implemented in several phase 1 studies to quantify the effects of antivascular agents on the tumor blood supply within hours to days after the start of treatment (110). Various analytic approaches have been used to quantify variables reflecting blood flow (F), vessel permeability surface area (PS), and the contrast distribution volume (generally assumed to reflect extravascular-extracellular space) from DCE-MRI data (111). Generally, treatment-induced changes in these variables reflect changes in F and/or PS (112). Substantial decreases in F and/or PS are induced both by vascular targeting agents (113–115) and by tyrosine kinase inhibitors (116,117). For VEGF-targeted agents, it appears that a substantial decrease in F and/or PS is necessary, but not sufficient for a significant reduction in tumor size (116,117). Interestingly, for vascular targeting agents, a similar reduction in F and/or PS is not associated with a reduction in tumor size. It is also worth noting that initial use of DCE-MRI in phase 1 studies were single-center studies, at sites

with considerable DCE-MRI expertise, raising concern about the ability to use this approach more generally (92). However, one recent study included three centers, without specific DCE-MRI expertise at all sites, demonstrating that the methodology can be standardized to yield consistent results in an early clinical trial at multiple institutions (116,118,119). In the study, DCE-MRI was used to help measure the pharmacodynamic response to acute dosing of AG-013736, a novel angiogenesis inhibitor, to identify suitable markers of biologic activity to assist in optimizing the dose and schedule of therapy. Thirty-six patients with advanced solid tumors were treated with various doses of AG-013736. In addition to standard measures of objective disease response and pharmacokinetic analysis, DCE-MRI scans were acquired at baseline and repeated cycle 1, day 2 after the scheduled morning dose of the AG-013736 in 26 patients. Indicators of a vascular response, such as the volume transfer constant (K^{trans}) and initial area under the curve (IAUC), were calculated to assess the effect of treatment on tumor vascular function. In this study evaluable vascular response data was obtained in 17 (65%) of 26 patients. An example of a scan in a responding patient with adenocarcinoma is shown in Figure 47-4. A linear correlation was found in which the percentage change from baseline to day 2 in K^{trans} and IAUC was inversely proportional to AG-013736 exposure (Figure 47-5). Using a conservative a priori assumption that a more than 50% decrease in K^{trans} was indicative of an objective vascular response, a 50% decrease in K^{trans} was achieved and corresponded to a plasma AUC_{0-24} of more than 200 (ng.hour/mL). Hence, although a sufficient decrease in tumor vascular parameters was observed at a dose chosen for further phase 2 testing by conventional toxicity criteria, the day 2 vascular response measured using DCE-MRI appears to be a useful indicator of drug pharmacology. Further research will be needed to determine if it is a suitable marker for predicting clinical activity.

FDG-PET uses an ^{18}F -labeled glucose analog (FDG) that is transported into cells by GLUT-1 and GLUT-3, phosphorylated by hexokinase and, since FDG-6-P is a poor substrate for glucose-6-phosphatase, there is little dephosphorylation and the radioactivity is trapped in the cell (120–122). Glucose metabolism is quantified as the activity in the tumor, normally restricted to the region of highest activity, relative to the amount of activity injected and patient's body weight, the so-called standardized uptake value (SUV). The potential for FDG-PET to assess drug-induced biologic effects prior to a change in tumor size was illustrated clearly in patients with advanced gastrointestinal stromal tumors treated with imatinib mesylate (123). Tumor FDG activity decreased markedly from baseline as early as 24 hours after a single dose of imatinib in all patients demonstrating a response by CT or MRI weeks later. Conversely, increased tumor FDG activity, activity at new sites, or both were seen in all patients with disease progression evident at a later date by conventional means. It should be noted that it has not been established whether these dramatic early decreases in FDG activity are due to decreased glucose metabolism (generally associated with viable tumor cells) or decreased glucose transport due to translocation of the glucose transporters from the cell membrane to the cytosol (124). Moreover, although a variety of treatment regimens result in reduced FDG activity following the

FIGURE 47-4 Representative dynamic contrast-enhanced magnetic resonance images from a patient with adenoid cystic carcinoma showing a decline in tumor perfusion after exposure to AG-013736. The tumor initial area under the curve (IAUC) values are mapped over the tumor region (shown quantitatively in the graphs below).



first cycle of therapy, after macrophage activity (which can result in increased FDG uptake) has subsided, yet before response is evaluable by standard methods (120), such dramatic effects are not generally observed so early after treatment. Nonetheless, FDG-PET shows considerable promise to provide an indication of decreased tumor viability prior to conventional methods and may provide a valuable downstream biomarker for biologic activity in phase 1 trials.

Although it is not reasonable to expect clinical efficacy in the advanced-stage patients entered into phase 1 trials and assessment of clinical response is not a primary focus of phase 1 trials, any indication that the drug/target impacts tumor growth is beneficial. Typically, tumor burden is assessed using computed tomography (CT) or MRI data. The method most commonly used to assess clinical effect is based on response evaluation criteria in solid tumors (RECIST), which was put forth in

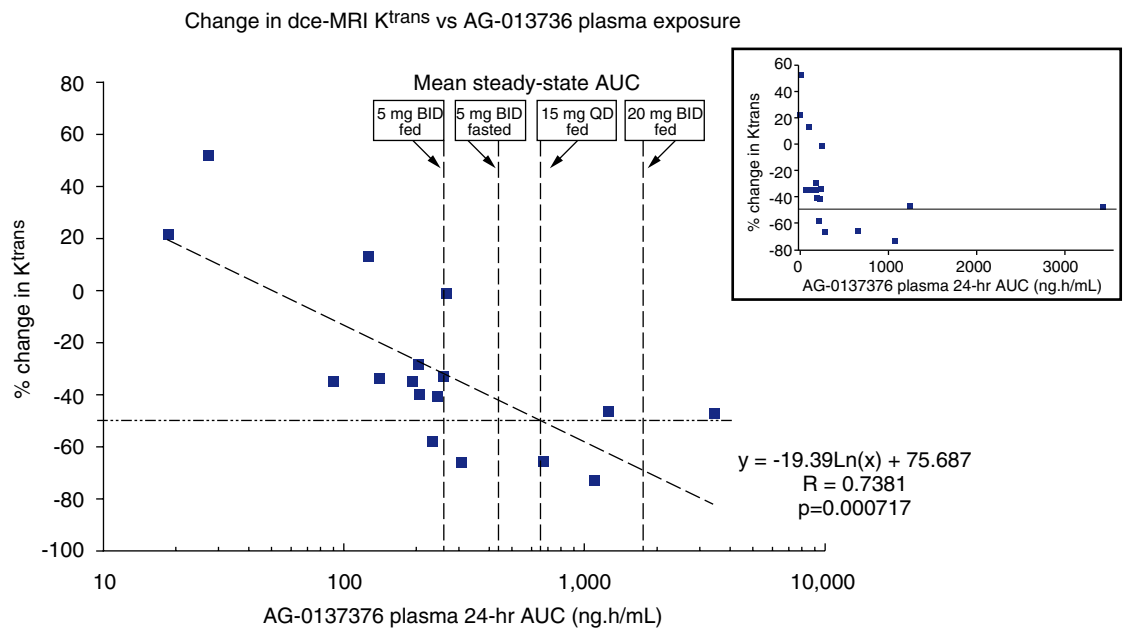


FIGURE 47-5 Correlation between plasma exposure of AG-013736 and change in K_{trans} in patients undergoing serial dynamic contrast-enhanced magnetic resonance imaging. The mean plasma concentrations of AG-013736 obtained in each dosing cohort are shown over the plot as a point of reference.

2000 as a simpler way to measure the response of tumors to experimental treatments (125). It should be noted that, in practice, RECIST are generally modified to address some of the concerns raised by the International Cancer Imaging Society (ICIS) regarding the strengths and weaknesses of using the RECIST criteria and what other issues should potentially be added to a response criterion (126). Nonetheless, even with these changes, concerns remain regarding RECIST especially in the context of early-phase trials (127). One point of particular concern is whether the single longest tumor dimension, determined in an axial plane, accurately represents changes in tumor burden since most tumors grow and regress irregularly (128). Another concern is how relevant the categoric response assessments (complete response, partial response, stable disease, and progressive disease), which were originally based on the error in oncologists' physical measurements of solid spheres arranged in random size order on a soft mattress and covered with a layer of foam rubber, are in the context of early-phase trials (129,130). It seems an alternative model, where response is considered a continuous variable, the change in tumor size (estimated as the single longest dimension, the cross-product of the longest dimension, and the perpendicular longest dimension or volume) after treatment (131), would be much more useful for evaluating clinical effect in phase 1 trials.

Conclusion

Cancer remains a major public health problem. Despite significant improvements in diagnosis, surgical techniques, general

patient care, and local and systemic therapies, most deaths from cancer are still due to metastasis that are resistant to conventional treatment. Novel therapeutic approaches are critically needed if we are to impact positively on patient outcome. Phase 1 studies are the critical link in targeting cancer, since they represent the first translation of years of laboratory/preclinical studies to the patient.

As drug development has evolved to a more tumor-targeted, or tumor-specific focus, so has the evolution of phase 1 trials moved from the more generic, mathematical modeling to the more rational design. In addition, it is increasingly being recognized that incorporation of select endpoints relative to patient eligibility in phase 1 trials are needed to more effectively and efficiently develop drugs clinically. Our current classification of most cancers is still based in large part on tissue type, tumor size, nodal status, and metastatic sites.

The future of tumor classifications is evolving as measurements such as molecular classifiers, genomics and proteomics become increasingly utilized not only preclinically, but also in the clinical arena to help better understand the molecular pathways that are aberrant in any given cancer cell (Figure 47-6). Several phase 1 designs are incorporating these tools, not so much as response predictors, but to help determine feasibility and to develop diagnostic/predictive tools for future clinical use. The hope is that a more personalized approach to clinical care will increase the efficacy of treatment, while decreasing its toxicity and cost. The result is the development of phase 1 trials aimed not only at defining dose and safety, but also at assisting in target validation. The latter will become increasingly important as we develop newer methods for targeting cancer.

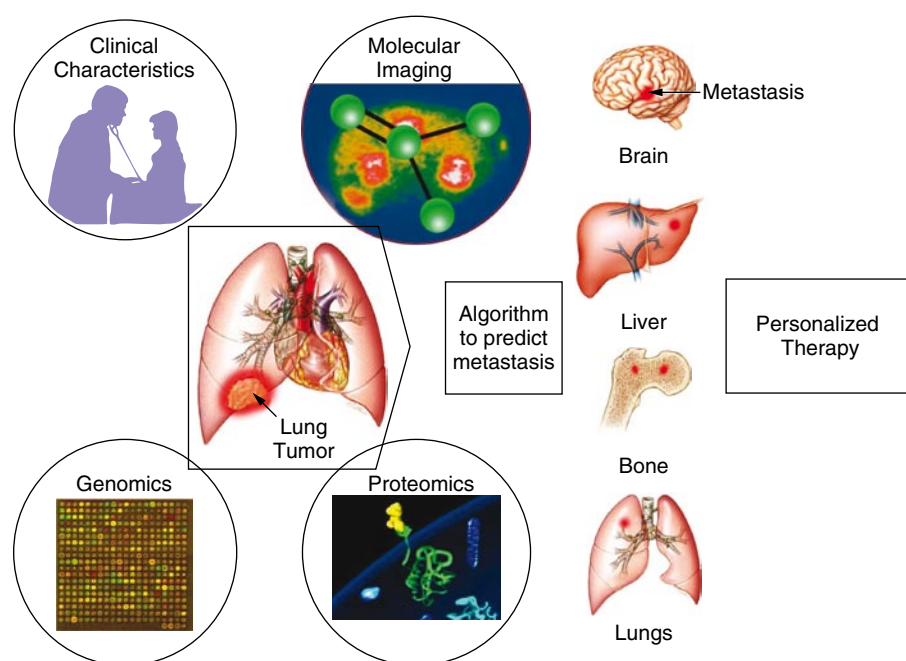


FIGURE 47-6 An algorithm of relevant proteomic, clinical, and imaging factors plus genomic factors all indicating a poor prognosis (metastasis) and best choice of molecular-targeted chemotherapy.

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Cancer Therapeutics

Cancer is characterized by the transformation of normal cells to ones characterized by abnormal cellular differentiation, proliferation, invasion, and metastases. The molecular and biochemical basis underlying the transformation process are becoming increasingly clear and provide critically important information for identifying new drug targets.

Normal cell division results from the interaction of growth factors with specific receptors (plasma membrane, cytoplasmic, or nuclear). This initiates a signal transduction cascade through receptor tyrosine kinases and downstream serine, threonine kinases that culminates in uncoiling of DNA by the action of histone acetylases and topoisomerases and activation of nuclear transcription factors that produce cell-proliferation and cell viability molecules. It should not, therefore, be surprising that cancer cells usurp these normal pathways, and that our most effective drugs target many of these processes (Figure 48-1).

Malignant cells acquire the ability to replicate indefinitely, invade, and metastasize. This process includes activation of telomerase, detachment from the primary site, anchorage-independent growth, invasion through the basement membrane, access to the blood or lymphatic vessels, entry to distant organs through adherence to visceral capillaries, escape from a variety of mechanisms designed to protect the host from “foreign invasion,” and the ability to grow in a foreign site. An understanding of the interactions between cancer cells and the surrounding stroma helped identify new targets to interfere with this characteristic of malignancy. Agents that target these processes include inhibitors of angiogenesis and metalloproteinases.

In this chapter, we attempt to place cancer chemotherapeutic drugs in a molecular biological context as summarized in Tables 48-1, 48-2, and 48-3.

Molecular Basis of the Therapeutic Index

Therapeutic index is defined as:

$$TI = \frac{LD_{50}}{ED_{50}}$$

where LD_{50} , or *median lethal dose*, is the dose of drug that causes death in 50% of experimental animals, and ED_{50} , or *median effective dose*, is the dose that produces a specified effect (“response”) in 50% of the population under study. The therapeutic index in the clinic similarly compares the dose of a drug that causes untoward toxicities to the dose that produces the desired therapeutic effect.

All drugs have targets, but it is the unique relationship of the target to the malignancy that can ultimately affect a drug’s therapeutic index. Traditional drug targets included DNA (nucleotide bases, enzymes of DNA synthesis, degradation, and repair), microtubules, and growth factor receptors. Newer targets include mutated or overexpressed oncogene products (EGFR [Her-1], Her-2/neu, ras, bcr:abl), tumor suppressor genes (*p53*, *Rb*), cell surface antigens (CD33, CD22, CD20, IL2-R), anti-apoptotic proteins (*bcl-2*), cell-cycle regulators (cyclin-dependent kinases), telomerase (a reverse transcriptase that allows continuous cell replication), and the machinery of protein synthesis (*t*-asparaginase) and degradation (ubiquitin proteasomal degradation). Drugs that affect these newer targets are often referred to as “targeted therapies,” creating the inaccurate impression that classic chemotherapeutics do not have targets. The specificity/selectivity of a drug for a particular target is in general proportional to the affinity constant, K_a , more accurately defined for competitive drug target interactions as the K_i , which is the reciprocal of the concentration of drug required to inhibit 50% of the target’s activity when controlled for all possible ligand or substrate concentrations.

There are several factors that help explain why cancer cells are more sensitive to cancer therapeutic drugs than normal tissues. For any drug, the TI is related to absorption, uptake, distribution and metabolism. Differences in tumor vasculature, intratumoral pressure, and drug binding may alter drug uptake in a favorable or unfavorable way. Classically, the therapeutic index of intravenously administered cancer chemotherapy has been thought to be due primarily to cell cycle kinetics. Since many chemotherapeutic agents are more effective against cycling than non-cycling cells and are tested under cell culture conditions where cancer cells rapidly proliferate (“log phase”), this *a posteriori* conclusion was derived from these observations. However, many solid tumors have a relatively long doubling time, yet a therapeutic index remains. Therefore, alternative explanations must exist. One includes differences in

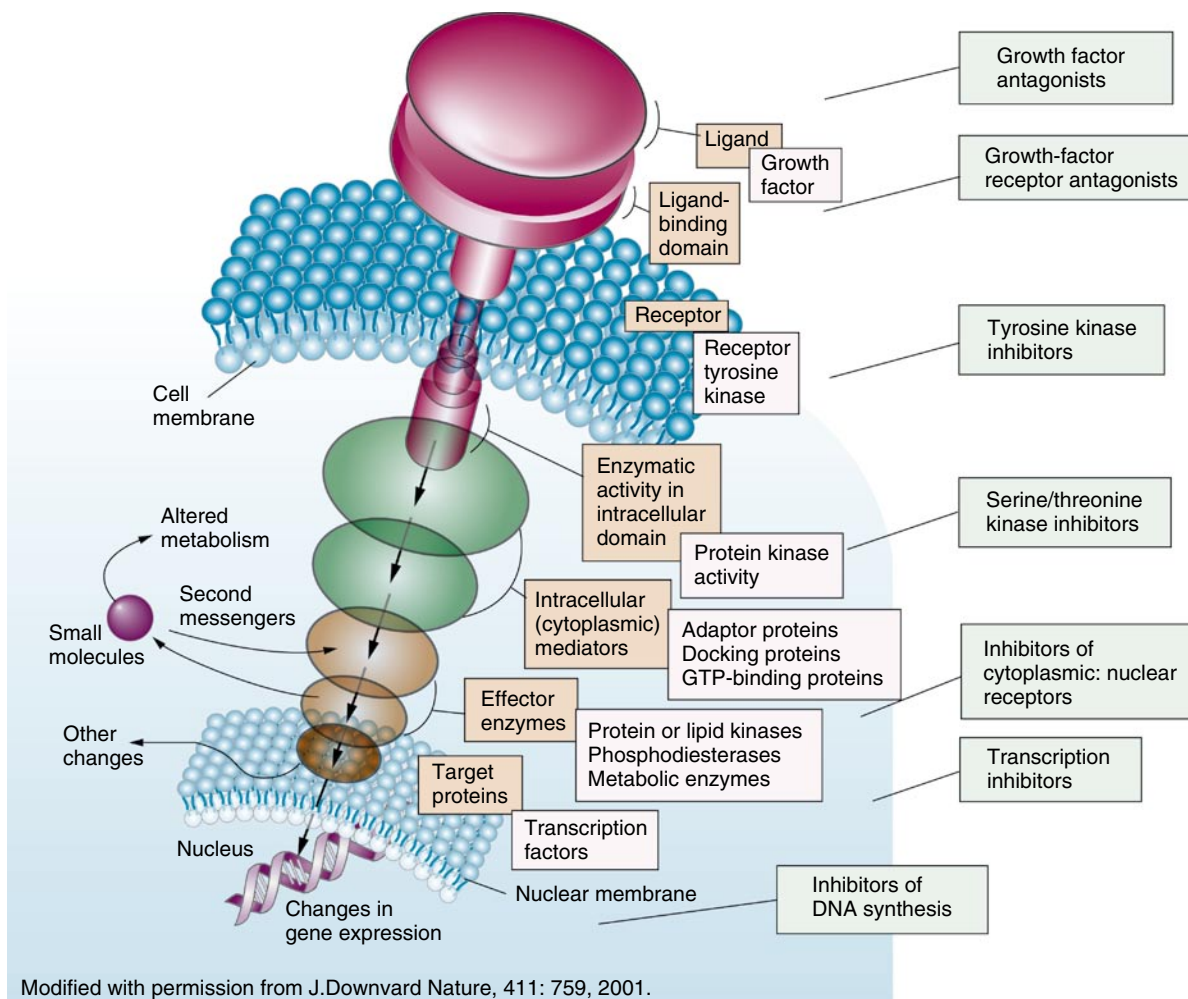


FIGURE 48-1 TARGETS FOR ANTICANCER DRUGS. Anticancer drugs work by interfering with the processes underlying normal cellular physiology. These include receptor activated signal transduction pathways culminating in transcriptional activation, DNA replication, protein synthesis and cell division. Reprinted by permission from Macmillan Publishers Ltd: J. Downward, *The ins and outs of signaling*, Nature 411:759, 2001.

energy requirements between normal and malignant cells. For example, whereas normal tissues utilize oxidative phosphorylation to metabolize glucose, tumors are often dependent on aerobic glycolysis. This is thought to reflect the pressure placed upon tumor cells to cope with relatively hypoxic conditions. Rather than utilizing the electron transfer chain within the mitochondria that yield 36 M of ATP per mol of glucose, cancer cells metabolize glucose via glycolysis, generating a net 2M of ATP per mol of glucose metabolized (the Warburg effect). As a result, cancer cells are metabolically fragile and are unable to cope as readily with cellular damage. This may be particularly relevant for drugs that block the effects of growth factors since growth factor depletion leads to rapid downregulation of nutrient transporters, which would lead to metabolic crisis in cancer cells more rapidly than in normal cellular counterparts.

Another concept that has stimulated renewed interest is that tumor cells exhibit "oncogene addiction" and that inhibition of one or more of these oncogene products rapidly results in apoptotic cell death in "addicted" cancer cells but not in normal counterparts. Thus tumor cells that depend on their survival by overexpression of a growth factor (e.g., EGF) would be more susceptible to inhibitors than normal cells.

Drugs Affecting Growth Factors and Growth Factor Receptors

Normal cell division results from the interaction of growth factors with specific receptors (plasma membrane, cytoplasmic, or nuclear). This initiates a signal transduction cascade culminating in activation of nuclear transcription factors that produce cell-proliferation and cell viability molecules. Thus, it stands to reason that some of our most effective drugs target growth factors or their receptors (Table 48-1) and the downstream consequences of this interaction that include activation of protein kinases, replication of DNA, transcription of mRNA, synthesis of new proteins, formation of the mitotic spindle through microtubule polymerization, and creation of interphase daughter cells via microtubule depolymerization (Table 48-2).

Drugs Affecting Growth Factors

Sex Hormones

Aromatase is an enzyme complex made up of two proteins, aromatase cytochrome P450 (CYP19) and NADPH-cytochrome

Table 48–1

Target	Examples	Use
Nuclear receptors Estrogen receptor Progesterone receptor Retinoid receptor Androgen receptor	Tamoxifen, toremifene, raloxifene, fulvestrant Megesterol acetate Retinoic acid Bicalutamide, flutamide, nilutamide	Treatment and prevention of breast cancer Treatment of breast cancer Treatment of promyelocytic leukemia Treatment of prostate cancer
Plasma membrane receptors		
Her-1 (EGFR) Her-2/neu Bcr:abl VEGF receptor cKit Flt-3 Fms Gonadotrophin receptors	Erlotinib, cetuximab, gefitinib Trastuzumab Imatinib Sorafenib, sunitinib Imatinib, sorafenib, sunitinib Sorafenib, sunitinib Sunitinib Abarelix, leuprolide, goselerin	Non-small cell lung; pancreas Breast Chronic myelogenous leukemia Renal cell Gastrointestinal stromal tumor (GIST)
Growth factors		
Aromatase inhibitors Vascular endothelial growth factor (VEGF)	Letrozole, anastrozole, exemestane Bevacizumab	Breast cancer Colorectal cancer
Multiple kinases		
VEGFR, PDGFR, cKIT, Flt-3, FMS, bRAF and Ret VEGFR, PDGFR, cRAF, bRAF, Flt-3, Fms	Sunitinib Sorafenib	Renal; GIST Renal
Miscellaneous		
CD20 CD52	Rituximab, zevalin, bexxar Alemtuzumab	Lymphoid malignancies Chronic lymphocytic leukemia (CLL)
DNA synthesis		
Dihydrofolate reductase	Methotrexate, trimetrexate,	Breast, lymphocytic leukemia choriocarcinoma, lymphoma
Thymidylate synthase Adenosine deaminase	5-fluorouracil, capecitabine, Pemetrexed Pentostatin, cladribine (2CDA)	Colon, breast, mesothelioma Hairy cell leukemia, lymphomas, CLL
DNA replication		
Nucleic acid bases Alkylating agents	• Nitrogen mustards (e.g., mechlorethamine, cyclophosphamide) • Nitrosoureas (e.g., BCNU) • Ethylenimines (e.g., thiotepa) • Alkyl Sulfonates (e.g., Busulfan) • Triazines (e.g., dacarbazine, temozolomide)	Leukemia, lymphoma, breast, brain, melanoma, etc.
Platinating agents	Cis-, carbo-, oxaliplatin	Lung, head and neck, bladder, germ cell, colorectal
Transcription inhibitors DNA methylation	Actinomycin-D 5'azacytidine	Wilms' tumor Myelodysplastic syndrome
Topoisomerases		
Topoisomerase I Topoisomerase II	Topotecan, irinotecan Doxorubicin, epirubicin, etoposide, mitoxantrone	Colorectal, ovary, lung, cervical Lymphoma, leukemia, lung, ovary, testicular
Microtubules		
Vinca alkaloids	Vincristine, vinblastine, vinorelbine	Breast, lung, acute lymphocytic leukemia (ALL), bladder, lymphoma
Taxanes	Paclitaxel, docetaxel	Breast, lung, bladder, ovarian
Protein synthesis		
	L-asparaginase	Childhood ALL, T-cell lymphoma
Protein degradation		
60S proteasome	Bortezomib	Multiple myeloma, mantle cell lymphoma

Table 48-2 Drugs That Alter Nucleic Acid Synthesis and Function

Target	Examples	Use
DNA Synthesis		
Dihydrofolate reductase	Methotrexate, Trimetrexate,	Breast, lymphocytic leukemia choriocarcinoma, lymphoma
Thymidylate synthase	5-fluorouracil, Capecitabine, Pemetrexed	Colon, breast, mesothelioma
Adenosine deaminase	Pentostatin, Cladribine (2CDA)	Hairy cell leukemia, lymphomas, CLL
DNA Replication		
Nucleic acid bases		
Alkylating agents	Nitrogen mustards (e.g., mechlorethamine, cyclophosphamide) Nitrosoureas (e.g., BCNU) Ethylenimines (e.g., Thiopeta) Alkyl Sulfonates (e.g., Busulfan) Triazenes (e.g., Dacarbazine, temozolomide)	Leukemia, lymphoma, breast, brain, melanoma, etc.
Platinating agents	Cis, carbo-, oxaliplatin	Lung, head and neck, bladder, germ cell, colorectal
Transcription inhibitors	Actinomycin-D	Wilms' tumor
DNA methylation	5'azacytidine	Myelodysplastic syndrome
Topoisomerases		
Topoisomerase I	Topotecan, Irinotecan	Colorectal, ovary, lung, cervical
Topoisomerase II	Doxorubicin, Epirubicin, Etoposide, Mitoxantrone	Lymphoma, leukemia, lung, ovary, testicular

P450 reductase. Inhibition of aromatase blocks the conversion of androgens (androstenedione) to estrone in peripheral tissues including fat, liver, muscle, and breast without detectable effects on adrenal synthesis of corticosteroids or aldosterone. Following the reports by Santen and colleagues that aminoglutethimide could inhibit the conversion of androstenedione to estradiol (2), aromatase became an attractive target for new drug development. Three new aromatase inhibitors are available for clinical use including letrozole (Femara), anastrozole (Arimidex), and exemestane (Aromasin). Whereas letrozole and anastrozole are reversible, non-steroidal inhibitors of aromatase, exemestane is a steroidal derivative of androstenedione that binds irreversibly to the enzyme and targets the protein for degradation. Aromatase inhibitors further deplete circulating estradiol in post-menopausal women and are

highly effective in the treatment of breast cancer in the adjuvant and metastatic settings (3). These well-tolerated medications produce osteopenia and are often prescribed with a bisphosphonate, calcium and vitamin D to prevent this complication.

Gonadotrophin-Releasing Hormones

Drugs that target receptors for gonadotrophin-releasing hormones (GnRHs) decrease the production of ovarian or testicular hormones. The most widely used agents (leuprolide, goserelin) are agonists of GnRH receptors that following immediate increase in gonadotrophins eventually produce castrate levels of sex hormones by the desensitization of GnRH receptors. A newer agent, Abarelix (Plenaxis), is a GnRH receptor antagonist that immediately decreases GnRHs without the disadvantage of an initial hormone surge.

Vascular Endothelial Growth Factor

The observation by Folkman and colleagues that tumors stimulate blood vessel formation, coupled with the knowledge that angiogenesis is required for tumor growth and metastasis, led to a search for effective inhibitors of this process (4). Vascular endothelial growth factor (VEGF) is produced by normal and neoplastic cells and regulates angiogenesis. Kim et al. (5) demonstrated that a murine monoclonal antibody versus VEGF inhibited growth of human cancer xenografts. This led to the development of bevacizumab, a human monoclonal antibody that binds to and inhibits VEGF preventing its interaction with VEGF receptors (Flt-1 and KDR), which are present on the surface of endothelial cells (6) and inhibits endothelial cell proliferation. Bevacizumab may also increase access of chemotherapy to cancer cells by decreasing the elevated interstitial pressures (7) seen in tumor masses.

Table 48-3 Drugs with Other Mechanisms

Target	Examples	Use
Microtubules		
Vinca alkaloids	Vincristine, Vinblastine, Vinorelbine	Breast, lung, ALL, bladder, lymphoma
Taxanes	Paclitaxel, Docetaxel	Breast, lung, bladder, ovarian
Protein Synthesis		
	L-asparaginase	Childhood ALL, T-cell lymphoma
Protein Degradation		
60S proteasome	Bortezomib	Multiple myeloma, mantle cell lymphoma

Bevacizumab is approved for first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy. Its most common side effects include hypertension, thrombosis, and proteinuria, but asthenia, gastrointestinal perforation, wound dehiscence, hemorrhage, and nephrotic syndrome have been reported, as has been a possible increase in congestive heart failure.

Drugs Affecting Growth Factor Receptors

Steroid Hormone Receptors

The interaction between steroid hormones and intracellular receptors recruit co-activators and corepressors to the nuclear transcription complex leading to the transcriptional activation of genes containing specific steroid response elements.

Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) bind with high affinity to cytoplasmic and nuclear estrogen receptors, then recruit transcriptional co-activators and corepressors to the transcription complex where they bind to estrogen response elements within promoter regions of estrogen-regulated genes. In certain tissues SERMs are antiestrogenic, whereas in others they are estrogenic; this is believed to be due to differential recruitment of co-repressors vs. repressors in a specific tissue type. For example, tamoxifen, behaves as an estrogen receptor antagonist in breast tissue, but as an agonist in the uterus, bone and liver. As a result, tamoxifen is a highly effective drug for the treatment of breast cancers that express hormone receptors but has the disadvantage of also increasing endometrial proliferation and the risk of uterine cancer. The estrogenic effect of tamoxifen on bone prevents osteopenia, while its estrogenic effects on the liver lowers cholesterol and increases the incidence of thrombosis. In contrast, raloxifene is a SERM that behaves as an antiestrogen in breast, uterus, and liver, but retains estrogenic activity in the bone. Raloxifene was disappointing for the treatment of metastatic breast cancer but is effective for breast cancer prevention and with fewer side effects than tamoxifen (8). Fulvestrant is a pure antiestrogen that works differently from the nonsteroidal SERMs. Fulvestrant binds to the estrogen receptor and targets it for ubiquitin-mediated proteasomal degradation.

Bexarotene (Targretin) is an analog of vitamin A that binds with high affinity to retinoid "X" receptors. It is approved for use against refractory cutaneous T cell lymphoma. Its side effects include cheilitis, headache myalgias, and arthralgias, as well as an increase in liver function tests, hypertriglyceridemia and hypercalcemia and hypothyroidism.

Drugs That Affect Oncogenic Growth Factor Receptors

Epidermal Growth Factor Receptor Family

Her-2/Neu. The discovery of a transforming element in the DNA of a glial cell line by Weinberg and colleagues led to the identification of HER-2/neu (9). When Slamon's group observed that HER-2/neu was overexpressed in certain aggressive breast cancers, a search ensued for a means to inhibit this membrane receptor (10).

The biology of HER-2/neu activation is complex with both putative and proven ligands. Intracellular signaling occurs after receptor activation that leads to the formation of hetero- and homodimers with other members of the EGFR receptor family including HER-1, HER-3, and HER-4.

Trastuzumab (Herceptin) is approved for use alone or in combination with paclitaxel for the treatment of metastatic breast cancers that overexpress HER-2/neu. In the 25% to 30% of patients who respond to treatment, a substantial number survive for long periods of time. Unfortunately, these patients often relapse in the central nervous system, probably due to the inability of trastuzumab to cross the blood-brain barrier rather than a predilection for these cells for the central nervous system (CNS). More recently trastuzumab was also shown to be highly effective in breast cancer patients receiving adjuvant chemotherapy (11).

Her-1 (epidermal growth factor receptor [EGFR]). EGFR is overexpressed in 60% to 80% of colorectal cancers and in many other tumor types. It can be transforming in laboratory models. The demonstration by Mendelsohn and colleagues that antibodies directed against the EGFR extracellular domain inhibited the growth of cancer cells led to the development of several types of EGFR antagonists (12). Cetuximab (Erbix) is a humanized mouse monoclonal antibody used in the treatment of colorectal cancer alone or in combination with irinotecan, and more recently in combination with the FOLFOX regimen. It has also shown promising activity in head and neck cancers and is synergistic with radiation in preclinical models. Cetuximab, like most other monoclonal antibodies, is extremely well tolerated. The major side effects include asthenia and an acneiform rash, the latter toxicity being a potential marker of response.

Several drugs have been developed to target the tyrosine kinase domain of the EGFR. For example, gefitinib (Iressa) received accelerated U.S. Food and Drug Administration (FDA) approval for the treatment of refractory NSCLC, but failed to show a survival advantage in larger randomized clinical trials. In contrast, erlotinib (Tarceva), a structurally similar molecule, produced a survival advantage in patients with NSCLC and is approved for use in this group of patients. Two reports demonstrated that gefitinib was particularly active in patients whose tumors harbored activating mutations in the tyrosine kinase catalytic domain of EGFR (13,14). This alteration was particularly prevalent in nonsmokers, women of Japanese decent, and tumors of bronchoalveolar histology. Erlotinib recently received approval in combination with gemcitabine for the treatment of pancreatic cancer. Acneiform rash and diarrhea are the most common side effects of these generally well-tolerated medications.

Bcr:abl. Of all the newer "targeted" therapies, none has been more impressive than imatinib (Gleevec; 4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate) for use in chronic myelogenous leukemia, gastrointestinal stromal tumors, and a chronic myeloproliferative disease characterized by eosinophilia (15). Imatinib targets the tyrosine kinase domain of the fusion protein formed by the reciprocal translocation involving the long arms of chromosomes 9 and 22 [t (9;22)(q34.1;q11.21)], the Philadelphia chromosome (Figure 48-2). It also is active against the tyrosine kinase activity of c-kit and the platelet-derived

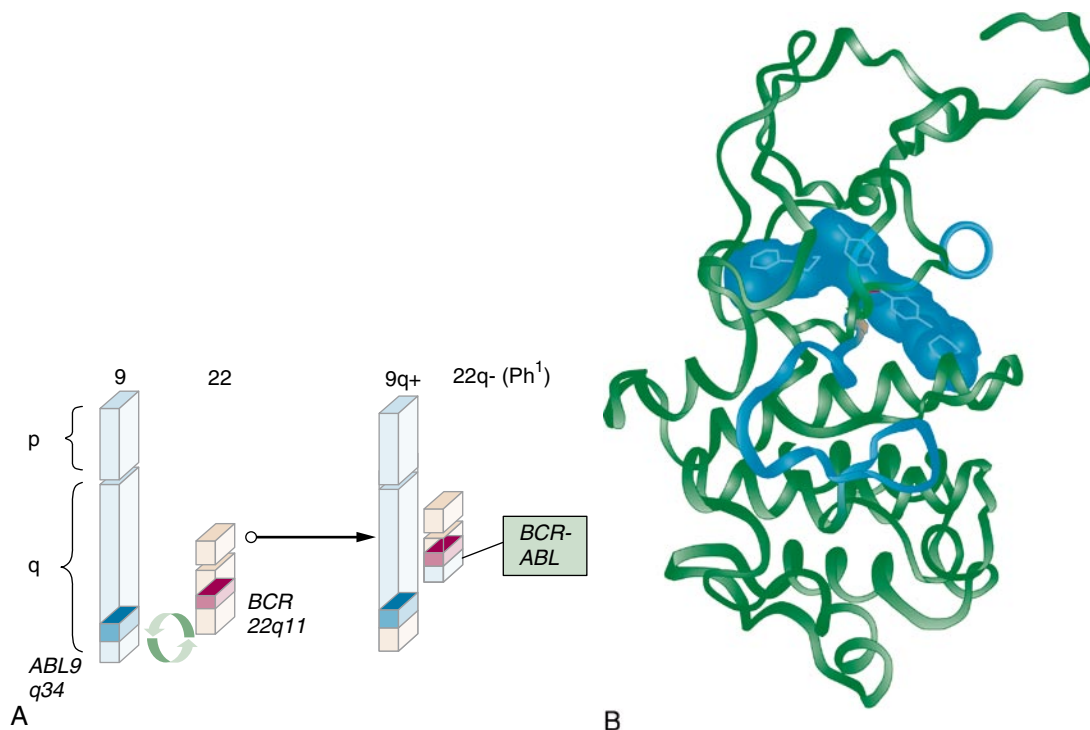


FIGURE 48-2 GENETIC AND STRUCTURAL CHANGES UNDERLYING THE ACTIVITY OF IMATINIB. **A:** The 9;22 chromosomal translocation that produces the bcr/abl oncogenic tyrosine kinase. **B:** Ribbon drawing of the structure of the Abl kinase domain (green) in complex with imatinib (Gleevec). The activation loops and the van der Waals surfaces corresponding to the inhibitor are colored. The DFG motif situated at the NH₂ terminus of the activation loop is shown in gold. Helix α C and the interlobe connector is shown in dark green. (From Ref. 16, with permission.)

growth factor receptor (PDGFR), the former accounting for its activity against GIST and the latter for its activity against the chronic myeloproliferative syndrome.

Denileukin, Diftitox, DAB389IL-2 (Ontak) is fusion protein consisting of cytotoxic A and B chain fragments (Met₁-Thr₃₈₇)-His of diphtheria toxin fused to IL-2 (17). It was approved under the FDA accelerated approval process for persistent or recurrent chronic T-cell leukemia (CTCL) expressing the CD25 component of the interleukin-2 receptor. Denileukin thereby delivers this potent exotoxin, which ADP ribosylates elongation factor-2 and terminates protein synthesis, to cutaneous T cell lymphoma cells and others cells expressing the IL-2 receptor. Side effects include flulike symptoms, acute hypersensitivity reactions, nausea and vomiting, vascular leak syndrome, infections, and transient elevation of liver function tests.

Drugs that target multiple signal transduction enzymes. Protein kinase inhibitors are rarely completely selective for a given target, given the similarities in the catalytic domains of these enzymes. Two drugs have recently been approved that have relatively permissive activities (i.e., they inhibit multiple kinases including those that phosphorylate tyrosines, serines, or threonines in substrate proteins). For example, sorafenib (Nexavar) was approved for the treatment of renal cell carcinoma and was initially believed to be specific for bRaf kinase, a serine/threonine kinase downstream of Ras that is mutated in melanoma and activated in several other malignancies. However, sorafenib is also a potent inhibitor of the VEGF receptor tyrosine kinases VEGFR-2 and 3, FLT-3, Kit, and PDGFR- β (18). Similarly, sunitinib (Sutent), which was recently approved for treatment of renal cell carcinoma and

for GIST patients who have progressed following treatment with imatinib, can also inhibit multiple kinases including PDGFR- α and - β , VEGFR 1,2, and 3, Kit, FLT-3, CSF-1 and RET (19). These drugs possess a different spectrum of untoward side effects than classical cytotoxic agents. For example, hypertension, bleeding (including tumor hemorrhage), diarrhea, mucositis, skin rash and taste abnormalities occur more frequently than for placebo. Sorafenib was associated with hand-foot syndrome (palmer plantar dysesthesia) and both drugs appear to increase cardiac events. For example, patients on sunitinib were more likely to have decreases in their left ventricular ejection fraction (10%) than placebo treated controls (1%), and those on sorafenib more likely to experience cardiac ischemic events (2.9%) than controls (0.4%).

Drugs That Alter Nucleic Acid Synthesis and Function

Growth factor/growth-factor receptor interactions activate signal transduction cascades that initiate DNA synthesis through transcriptional activation of cell proliferation genes culminating in DNA replication and cell division. A good example is phosphorylation of the retinoblastoma protein (Rb) by CDK4 and CDK6 followed by CDK2. This releases a family of bound transcription factors, E2F(s), which activate genes critical for progression into S phase, and are overexpressed in the malignant phenotype (e.g., thymidylate synthase, dihydrofolate reductase). For DNA to then be transcribed it must be made accessible to transcription factors. This is accomplished by releasing DNA from histone packaging

via histone acetylases and unwinding of the double helical structure via the action of helicases and topoisomerases. Therefore, it is not surprising that some of our most effective chemotherapeutic drugs target these downstream events, including DNA synthesis, transcription, and topoisomerase activities.

Inhibitors of Nucleic Acid Synthesis

Dihydrofolate reductase (DHFR), the enzyme that replenishes reduced folate pools, was one of the earliest targets for cancer chemotherapy drugs that included aminopterin and methotrexate. By inhibiting DHFR, methotrexate and its polyglutamated derivatives deplete reduced folates and thereby block the synthesis of thymidylate and de novo purine synthesis. Similarly, targeting thymidylate synthase (TS) to interfere with DNA synthesis led to the development of 5-fluorouracil and 5-fluorodeoxyridine; these drugs remain critically important in the modern oncologist's armamentarium.

Pemetrexed (Alimta) is a unique antifolate containing a 6-5 fused pyrrolo[2,3-d]pyrimidine nucleus that inhibits thymidylate synthase, glycinamide ribonucleotide formyltransferase (GARFT) and dihydrofolate reductase, folate-dependent enzymes involved in the synthesis of thymidine and purine nucleotides. Like methotrexate, pemetrexed is transported into cells by the reduced folate carrier and membrane folate-binding proteins where it is metabolized to polyglutamates by folylpoly- γ -glutamate synthetase. Polyglutamated forms are retained intracellularly and have greater affinity for thymidylate synthase (TS) and GARFT than pemetrexed monoglutamate. Pemetrexed also inhibits DHFR. Therefore, this drug interrupts de novo synthesis of thymidine and purine nucleosides (20). Pemetrexed is approved for the treatment of mesothelioma and second line treatment of non-small cell lung cancer, and has shown promising activity in the treatment of breast cancer in combination with gemcitabine. Pretreatment with folic acid and vitamin B₁₂ is now used to ameliorate the most frequent side effects, including bone marrow suppression, fatigue, and skin rash.

Capecitabine (Xeloda) is an orally administered carbamate derivative of 5'-deoxy-5-fluorouridine that acts as a prodrug of 5-FU (21). It is approved for use as a single agent in metastatic breast cancer that is resistant to anthracyclines and taxanes and in combination with docetaxel for metastatic breast cancer after relapse from anthracyclines. It is also approved as a single-agent in the first-line treatment of metastatic colorectal cancer. Capecitabine is converted to 5'-deoxy-5-fluorocytidine (DFCR) by carboxylesterases in the liver. DFCR is then converted to 5'-deoxy-5-fluorouridine (DFUR) by cytidine deaminase in the liver and tumor tissue. DFUR is then converted to 5-FU by thymidine phosphorylase. The therapeutic index of capecitabine may be based on an increased activity of thymidine phosphorylase in tumor compared to normal tissues; therefore, capecitabine may have additional selectivity over 5-FU, although this has not been rigorously demonstrated in the clinic. Its side effects, neutropenia, diarrhea, stomatitis, and palmer-plantar dysesthesia (hand-foot syndrome), are similar to those seen when 5-FU is given by continuous intravenous infusion.

Gemcitabine (Gemzar) is the 2'-deoxy-2',2'-difluorocytidine analog of deoxycytidine, which was selected for development because of its activity against murine solid tumors (22). It is approved for the treatment of recurrent pancreatic cancer and for front-line treatment of inoperable, locally advanced, or recurrent/metastatic NSCLC. Gemcitabine inhibits DNA synthesis by intracellular conversion by deoxycytidine kinase to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides that lead to competitive inhibition of DNA polymerase. In addition, gemcitabine diphosphate inhibits ribonucleotide reductase, thereby blocking the synthesis of deoxynucleoside triphosphates for DNA synthesis. Gemcitabine triphosphate also competes with dCTP for incorporation into DNA. The reduction in the intracellular concentration of dCTP (by the action of the diphosphate) enhances the incorporation of gemcitabine triphosphate into DNA (self-potential). Side effects include myelosuppression, nausea, vomiting, transaminitis, diarrhea, stomatitis, proteinuria, hematuria, fever, maculopapular rash, peripheral edema, and flu-like symptoms.

Inhibitors of DNA Topoisomerase

Topoisomerases correct the altered DNA topology that occurs during DNA replication and transcription by inflicting transient single-strand (topoisomerase I) or double-strand (topoisomerase II) breaks in DNA. Several natural-product antineoplastic drugs inhibit topoisomerase I or topoisomerase II. The effect of drugs on topoisomerases is different than most other enzyme inhibitors (i.e., they "poison" the enzymes by inhibiting religation of the DNA nicks produced during topoisomerase catalysis, thereby locking the enzyme in the "on" or catalytic conformation) (23).

Topoisomerases are increased during S phase, and cancer cells appear to have greater topoisomerase activity than their normal counterparts. Thus topoisomerase-targeting drugs inflict greater drug-induced DNA damage and cell death to cancer cells than normal cells. Differences in the processing of topoisomerase-mediated DNA damage by malignant vs. normal cells may also be important in the therapeutic index of these drugs.

Currently approved topoisomerase II inhibitors such as doxorubicin, daunomycin, mitoxantrone, etoposide, and teniposide are part of many combination chemotherapy regimens and form the basis for some of our earliest curative regimens (e.g., non-Hodgkin lymphoma [doxorubicin], acute myelogenous leukemia [daunorubicin], and germ cell malignancies [etoposide]). Additional topoisomerase II formulations include liposomal doxorubicin (Doxil), originally approved in 1995 for Kaposi sarcoma (Doxil is now approved for relapsed ovarian cancer) and epirubicin (Ellence), an anthracycline that is approved as a component of adjuvant therapy for node-positive breast cancer.

Currently approved topoisomerase I inhibitors include the camptothecin-derivatives topotecan and irinotecan. Camptothecin was identified in the early 1960s by Wani and Wall as a potent anticancer alkaloid present in extracts from the *Camptotheca acuminata* (Chinese yew) tree (24). The sodium salt entered clinical trials in the 1970s but was discontinued due to severe and unpredictable toxicities. The appreciation of its unique mechanism

of action by the Liu laboratory (23) and the pH dependence of lactone ring cleavage led to development of more stable and safer derivatives. Topotecan, a semisynthetic analog of camptothecin produced by adding a basic side chain at the 9-position of the A-ring of 10-hydroxycamptothecin, has increased water solubility without requiring hydrolysis of the lactone (E-ring). Topotecan was the first topoisomerase I inhibitor approved for clinical use and is indicated for refractory ovarian cancer and “sensitive” small cell lung cancer after first-line chemotherapy. Its major untoward side effects include myelosuppression, fatigue, moderate nausea and vomiting, diarrhea, and alopecia.

Irinotecan (Camptosar; CPT-1) is another semisynthetic analog of camptothecin approved in 1996 for the treatment of colorectal cancer refractory to 5-FU. It is a prodrug that is converted to the active compound (SN-38) by carboxylesterases (which are present at relatively high concentrations in the intestine). Indications now include first-line treatment in combination with 5-FU and leucovorin for metastatic colon or rectal cancer and for colorectal cancer that has progressed or reoccurred following initial treatment with 5-FU. Its myeloid and gastrointestinal toxicities are enhanced when given in combination with 5-FU and leucovorin. Patients with Gilbert’s disease or other polymorphisms in the UGT1A1 glucuronyl transferase have an increased risk of toxicity (25). Recently, the combination of irinotecan and 5-FU/LV was approved in combination with bevacizumab for first line treatment of metastatic colorectal cancer.

Alkylating Agents

DNA replication requires that the bases be accessible for Watson-Crick pairing. This normal biochemistry is interrupted by the alkylating agents (26). The work of Alfred Gilman, and their colleagues at Yale University demonstrated that nitrogen mustard could abolish lymphomas in experimental animals and had chemotherapeutic effects in man. These studies carried out during World War II, were the start of cancer chemotherapy. The classes of alkylating agents include the following:

- Nitrogen mustards (e.g., mechlorethamine, cyclophosphamide)
- Nitrosoureas (e.g., BCNU)
- Ethylenimines (e.g., Thiopeta)
- Alkyl sulfonates (e.g., Busulfan)
- Triazenes (e.g., Dacarbazine, temozolomide)

The newest alkylating agent, temozolomide (Temodar), is the 3-methyl derivative of mitozolomide, which was discovered when screening a series of 1,2,4-triazenes and triazinones synthesized in the 1960s and 1970s (27). Mitozolomide was the most promising compound but it had severe and unpredictable side effects. Temozolomide, like DTIC, methylates N-7 and O’6 of guanine to produce the cytotoxic lesion. Temozolomide is approved the treatment of adult patients with refractory anaplastic astrocytoma (disease progression after nitrosourea or procarbazine) and as adjuvant treatment when given with radiation to patients with glioblastoma multiforme. The most common side effects are lymphocytopenia, transaminitis, nausea and vomiting, hyperglycemia, anemia, and thrombocytopenia.

Platinating Agents

The therapeutic properties of cisplatin were deduced from studies of bacterial motility in electric currents, where bacterial cell death was observed adjacent to platinum electrodes (28). Platinating agents consist of platinum complexed with ligands that are displaced by nucleophilic attack to produce inter- and intrastrand DNA adducts. Currently approved platinating agents include cisplatin, carboplatin, and oxaliplatin, which differ in their spectrum of activity and untoward side effects. For example, cisplatin is active against lung cancer, head and neck cancer, and in combination with vinblastine and bleomycin led to the first reliable cures of testicular cancer. Cisplatin is highly emetogenic and is a potent oto- and nephrotoxin. In contrast, carboplatin appears to be less active than cisplatin against tumors of the head and neck and perhaps non-small cell lung cancer. It is less emetogenic and nephrotoxic than cisplatin, but more myelotoxic. Oxaliplatin (Eloxatin) (*trans*-1,2-diaminocyclohexane oxalatoplatinum) is a divalent coordination complex of platinum consisting of an oxalato group and a 1,2-diaminocyclohexane (DACH) ligand. This third generation platinum derivative is more active than cisplatin against colon cancer. Unlike *cis*- or carboplatin, renal dysfunction, ototoxicity, and alopecia are uncommon. The DLT is peripheral neuropathy, which can be acute (lasting less than 14 days) or persistent (14 days or greater); an acute syndrome of pharyngolaryngeal dysesthesia is often exacerbated by exposure to cold (temperature, objects, or liquids). Oxaliplatin is approved for use in combination with 5-FU/LV for the treatment of metastatic colorectal cancer. The activity of oxaliplatin is dependent on the formation of intrastrand (Pt)-DNA adducts/cross links, similar to that of other Pt compounds. DNA cross-links inhibit replication and transcription and activate apoptosis. Oxaliplatin has been reported to also down-regulate TS and increase sensitivity to fluoropyrimidines (29). Oxaliplatin forms two- to tenfold less Pt-DNA adducts than cisplatin at equimolar and equitoxic concentrations suggesting that these adducts may be more cytotoxic or that alternative mechanisms of action exist. DACH-Pt-DNA adducts formed by oxaliplatin are bulkier and more hydrophobic than *cis*-diamine-Pt-DNA adducts and may have greater inhibitory effects on DNA repair. DNA mismatch repair complexes do not recognize DACH-Pt-DNA adducts.

Drugs That Affect the Mitotic Apparatus

Vinca Alkaloids and Taxanes

Microtubules are essential for normal cellular function (30). They form the mitotic spindle, maintain cell shape, organize the location of organelles, mediate intracellular transport and secretion, and neurotransmission as well as axonemal flow and cell motility. Microtubules are composed of α - and β -tubulin dimers organized in bundles of 13 protofilaments that form hollow cylinders. The protofilaments are aligned with the same polarity. The (+) or fast-growing end moves outward from the nucleus to the plasma membrane, whereas the (–) or

slow-growing end marks the site of nucleation of the microtubule, which often begins in the centrosome. Time-lapse microscopy demonstrated that microtubules grow in spurts or may disappear altogether. This process, termed “dynamic equilibrium,” is an essential feature of microtubule physiology. For microtubules to elongate they require the addition of both α - and β -tubulin bound to GTP. Once bound to GTP, β -tubulin forms a GTP cap at the elongating end. Rapid microtubule growth requires bound GTP to increase the affinity for other tubulin molecules. During depolymerization, GTP is hydrolyzed more rapidly than it can be added, resulting in weakening of the bonds that hold the tubulin molecules together.

Antimitotic drugs act by interfering with the normal dynamic equilibrium of microtubules, thereby disrupting the function of the mitotic apparatus. In addition, by affecting microtubules in interphase cells, these drugs inhibit cell motility and normal subcellular organization.

Drugs Affecting Microtubule Polymerization

Paclitaxel (Taxol) was isolated in 1971 by Wani and Wall as an active moiety from the bark of the pacific yew, *Taxus brevifolius* (31). The taxanes are large alkaloid esters consisting of a taxane ring linked to a four-member oxetan ring at positions C-4 and C-5. Docetaxel (Taxotere), a semisynthetic derivative produced from 10-deacetylbaccatin III, is more water soluble and more potent in vitro. Initially, the difficulties encountered in formulating this insoluble compound and its toxicities in patients diminished enthusiasm for developing this new agent. However, interest in paclitaxel was renewed when the Horowitz laboratory identified its unique mechanism of action (i.e., stabilization of polymerized microtubules; 32). Paclitaxel and docetaxel share broad-spectrum antitumor activity including breast, lung, ovarian, and bladder cancers. Both have effects against lymphoid malignancies. Abraxane is a newly derivatized formulation of paclitaxel bound to albumin nano-particles, allowing the drug to be administered at a higher MTD and without Cremophor-EL, thereby eliminating the need for extensive premedication.

Taxanes preferentially bind to the N-terminal 31 amino acids of the β -subunit of tubulin oligomers or polymers and inhibit microtubule depolymerization. At nanomolar concentrations, the taxanes produce a mitotic block without increasing microtubule polymer mass. At stoichiometric concentrations (1 M drug per 1 M tubulin dimer), taxanes polymerize and stabilize microtubules in the absence of GTP or microtubule associated proteins (MAPs); these microtubules are resistant to depolymerization by calcium or low temperature.

The taxanes have broad-spectrum anticancer activity. They are used predominantly in the treatment of solid tumors (ovarian, breast, lung, bladder, head, and neck). Although docetaxel is more potent than paclitaxel, there is little direct evidence that it is more effective. Recent data suggest that combination of docetaxel with estramustine or prednisone may prolong survival of patients with hormone-refractory prostate cancer (33). Preclinical studies demonstrated that unlike most chemotherapeutic agents, taxanes are

more active against cancer cells harboring p53 mutations; this may help explain their widespread activity and therapeutic index (34).

Both paclitaxel and docetaxel produce peripheral neuropathy, dose-limiting bone marrow suppression, and alopecia. Nausea, vomiting and diarrhea occur with both drugs, but are rarely severe. Docetaxel can cause vascular permeability (peripheral edema, pleural effusions, and ascites). Fluid retention occurs at cumulative doses above 400 mg/m² and may be decreased by lower single doses (<60–75 mg/m²) or premedication with dexamethasone. Docetaxel also produces skin toxicities including an erythematous maculopapular rash of the forearms and hands. Both paclitaxel and docetaxel cause type I hypersensitivity reactions characterized by flushing, bronchospasm, dyspnea, and hypotension.

Drugs Affecting Microtubule Depolymerization

The vinca alkaloids were identified as extracts from the pink periwinkle plant (*Catharanthus roseus*, G. Don) that produced granulocytopenia in rats. This observation led to the isolation of four active alkaloids, of which two, vincristine and vinblastine, became active therapeutic agents. Today, this class also includes vinorelbine and vindesine (35).

The vinca alkaloids are large symmetrical molecules consisting of a dihydroindole nucleus (vindoline) connected to an indole nucleus (catharanthine) by a methylene bridge (Figure 48-3). Vincristine and vinblastine differ by a single R1 substituent, whereas vinblastine and vindesine differ in the R2 and R3 positions; vinorelbine has a modification of the catharine ring.

The mechanism of action of these drugs is concentration related. At substoichiometric concentrations, they bind to high-affinity sites at the ends of microtubules (K_a 5.3×10^{-5} M) and prevent microtubule polymerization. At higher concentrations, vincas bind to low-affinity, high-capacity sites (K_a $3\text{--}4 \times 10^{-3}$ M) and lead to the disintegration of formed microtubules.

Despite structural similarities, the spectrum of activity and toxicities of the vinca alkaloids are different. For example, vincristine is highly effective against non-Hodgkin lymphoma, Hodgkin's disease, and pediatric solid tumors, yet vincristine has little activity against adult solid tumors. In contrast, vinorelbine is active against breast and lung cancer. Vinblastine is most frequently used in the treatment of testicular cancer and non-Hodgkin lymphoma and is an active agent in the treatment of breast cancer. Vinca alkaloids are potent inhibitors of angiogenesis, which may contribute to their activity (36).

Major toxicities include dose-limiting myelosuppression and neurotoxicity. Vinblastine and vinorelbine produce far greater neutropenia than vincristine, with nadirs occurring at 4 to 10 days with recovery seen in most patients by 7 to 21 days. All three agents cause mild alopecia and are severe vesicants. Vinorelbine may cause chest pain and other deep-seated pain of unspecified origin. Respiratory reactions include acute bronchospasm and subacute cough dyspnea and pulmonary infiltrates have also been reported and appear responsive to steroids. The most frequent neurotoxicities are numbness and tingling of the extremities, loss of deep tendon reflexes, and distal muscle weakness. Although the

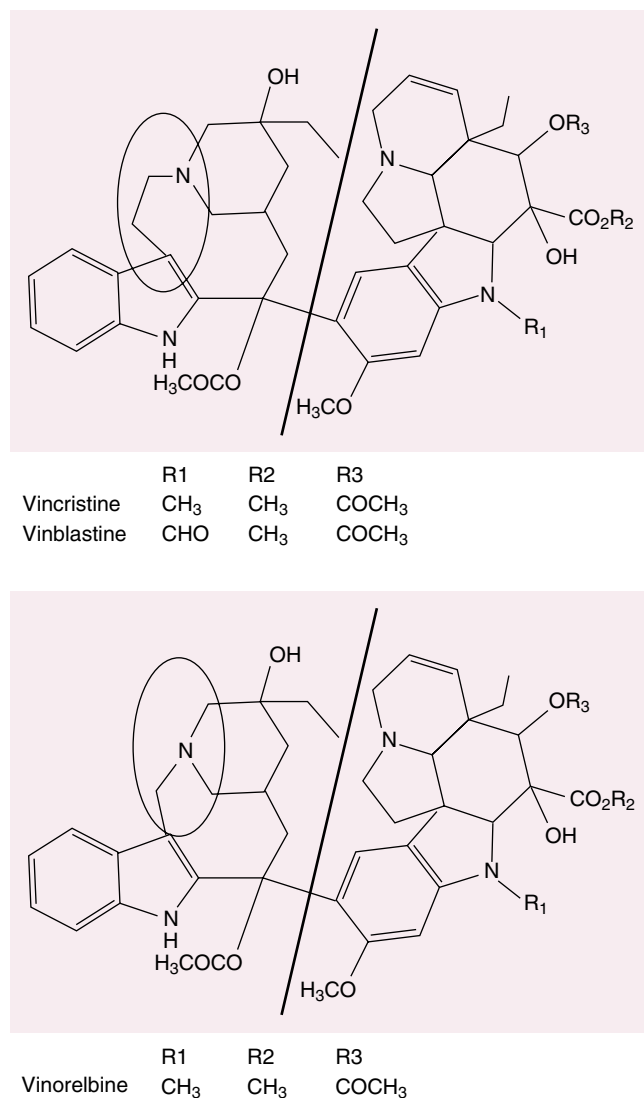


FIGURE 48-3 STRUCTURES OF VINCA ALKALOIDS. The slanted line demarcates the catharanthine (left of the line) and indoline (right of the line) nuclei that are connected in these drugs. Substituents that differ among the compounds are shown. The ellipse indicates the modification in the catharanthine ring in vinorelbine relative to the other compounds.

sensory changes are bothersome, they usually reverse over time and may not require discontinuation of the drug. Loss of motor function is a later and more ominous side effect, requiring discontinuation of the medication and or a search for other contributing factors.

Drugs That Affect Protein Synthesis and Degradation

Few drugs affecting protein synthesis and/or degradation have been used for the treatment of cancer. This is somewhat surprising given that the complexity of the process provided important targets for the development of antibacterial antibiotics. The process of protein synthesis includes initiation, elongation, and termination requiring an array of amino acids, structural proteins, enzymes and substrates including ribosomes, initiation factors, elongation factors, termination factors, and protein kinases that regulate the function of many of these elements. Historically, L-asparaginase has been the prototype of this class of drug. Recent attention has turned to the factors that control protein degradation. The ansamycins (e.g., geldanamycin) represent a class of agents that interfere with protein chaperones (i.e., proteins that bind to and stabilize newly formed or damaged proteins). Proteins targeted for degradation due to damage, improper folding, or cellular excess may proceed through one of two pathways, lysosomal or proteasomal. Bortezomib (Velcade), a drug that broadly inhibits proteases that are present in the proteasome, has been approved for the treatment of refractory myeloma (37). It interferes with proteasomal degradation of proteins such as the NF- κ B inhibitor, I κ B. NF- κ B is a constitutively activated transcription factor in myeloma (38) shown to promote oncogenesis by increasing growth factors (IL-6, VEGF), cellular adhesion molecules (ICAM1, VCAM1), and anti-apoptotic proteins (bcl-2, IAP). For a drug that interferes with a fundamental cellular process, bortezomib is relatively well tolerated with peripheral neuropathy being the most troublesome side effect.

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Natural and Acquired Resistance to Cancer Therapies

Chemotherapy of cancers has resulted in some notable successes, such as the cure of the majority of patients with childhood leukemias, testicular carcinomas, and Hodgkin and non-Hodgkin lymphomas. In other cancer types, such as breast, colorectal, and lung, chemotherapy also cures some patients when used in the adjuvant setting, that is, after debulking of the cancers with surgery and/or radiotherapy. However, for these common epithelial cancers, cure is rarely attained when the cancers have reached the stage of known metastatic disease. In such cases of advanced-stage cancers, chemotherapies may provide clinical benefit in the form of temporary remission of tumors and abatement of symptoms. In most patients, resistance to therapies is manifested as growth of cancers despite administration of drugs, or in relapse or regrowth after a remission. In this chapter, we review reasons for failure of systemic cancer therapies (Table 49-1).

Pharmacologic and Physiologic Causes of Treatment Failure

Inadequate Drug Dose

In addition to cellular mechanisms of drug resistance, pharmacologic and physiologic factors may play a role in outcomes of cancer therapy. Pharmacologic factors include inadequate drug dosing or suboptimal scheduling of agents (1–3). Traditionally, cytotoxic drugs have been administered at the maximum tolerated doses based on toxicities to normal tissues. Increased therapeutic efficacies with increasing doses have been demonstrated for virtually all cytotoxins in preclinical models, and are supported by clinical data relating dose to efficacy in many cancers (3).

For recently developed targeted drugs, such as kinase inhibitors, the optimal dose may not be the maximum tolerated dose in patients. The relationships between antitumor efficacy, side effects, pharmacodynamic endpoints, and dose of these agents are complex and require novel methodologies and study designs.

Suboptimal Schedule of Drug Administration

With regard to schedule, drugs with short half-lives and cell cycle phase-specific mechanisms of action are typically more active with

continuous drug exposure schedules, such as repeated dosing and continuous infusions (1). Examples include the antimetabolites cytarabine and 5-fluorouracil, the antibiotic bleomycin, and topoisomerase I inhibitors.

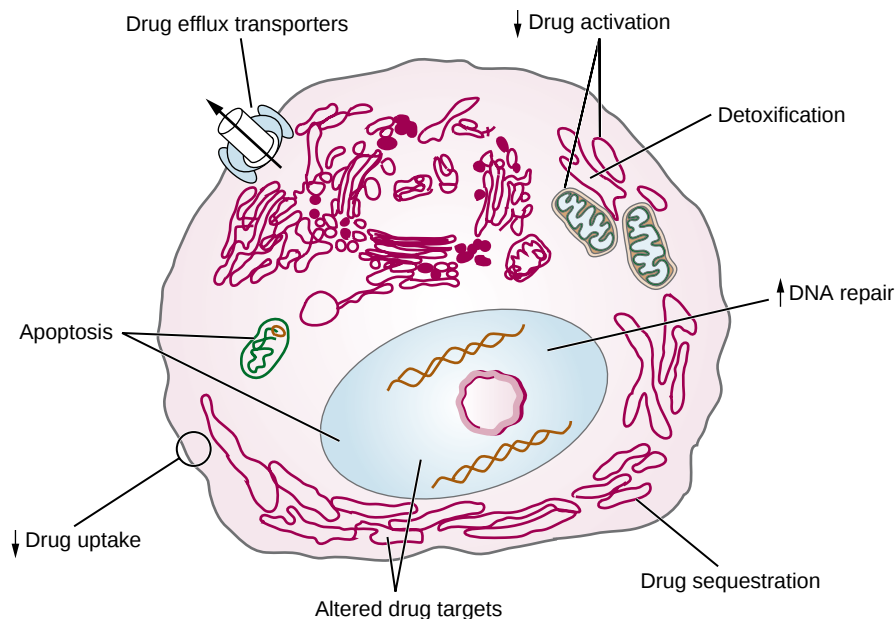
Drug Sanctuary Sites (Central Nervous System and Testis)

A physiologic cause for drug resistance is inadequate distribution of drugs to the central nervous system (CNS) and testis, because of the blood-brain and blood-testicular barriers. A major component of these barriers is endothelial cell expression of the multidrug transporter, P-glycoprotein (4,5). Thus, relapse in the CNS and testis

Table 49-1 Causes for Failure of Systemic Cancer Therapies

A.	Pharmacologic and physiologic mechanisms
1.	Inadequate drug dosing
2.	Suboptimal schedule of drug administration
3.	Sanctuary sites (blood-brain and blood-testicular barriers)
4.	Poor diffusion and distribution into tumor tissues
B.	Cellular mechanisms of drug resistance
1.	Drug efflux transporters
2.	Impaired drug uptake
3.	Mutation or altered expression of molecular targets
4.	Intracellular redistribution of drug
5.	Detoxification of drug or intermediate drug product
6.	Enhanced DNA repair
7.	Decreased drug activation
8.	Altered pathways for programmed cell death (apoptosis)

FIGURE 49-1 Mechanisms of cellular drug resistance.



became evident as major sites of treatment failure after most children with acute lymphoblastic leukemia achieved a clinical complete remission. New treatment strategies such as high-dose methotrexate and intrathecal administration of chemotherapy were devised to circumvent the problem of relapse in these so-called sanctuary sites.

Poor Drug Diffusion into Cancer Tissues

The physiology of abnormal new blood vessel formation (angiogenesis), and areas of poor blood supply, may also limit the ability of anticancer drugs to distribute into cancer tissues, and thus result in treatment failure (6,7).

Cellular Mechanisms of Drug Resistance

Intrinsic versus Acquired Resistance

The various types of cellular mechanisms of drug resistance are depicted in Figure 49-1. These mechanisms can be either intrinsic (i.e., constitutively expressed in the tumor tissue from the outset) or acquired (i.e., derived by mutation or induction of gene expression within the tumor cell population), often after exposure to therapies (8,9). Intrinsic resistance generally refers to the preexisting expression of cellular defense mechanisms, which are also present in the normal tissues from which the cancer is derived.

Epithelial tissues such as the colon, kidney, and liver express many transporters and detoxifying enzymes for xenobiotics. These defense mechanisms are also usually expressed in carcinomas originated from those organs, and confer resistance to many chemotherapeutic drugs. Normal hematolymphoid tissues are less well defended against xenobiotics, with the exception of certain subtypes (natural killer cells, hematopoietic stem cells [10]). Thus, lymphomas and leukemias express fewer resistance mechanisms and are generally more sensitive to chemotherapies than epithelial cancers.

The distinction between intrinsic and acquired resistance is blurred in the case of oncogenic mechanisms, such as mutations of *p53* and translocations resulting in high activity of the anti-

apoptotic protein, BCL-2. These oncogenic mutations result in the inhibition of normal apoptotic mechanisms, and thus have a dual effect of causing cancer and resistance to anticancer therapies, which require apoptotic signaling to kill cancer cells (11–24). The tissue microenvironment of cancers, involving cell-cell and cell-matrix interactions, may also lead to resistance to cancer therapies via pathways which inhibit apoptosis (25).

Genetics of Drug Resistance

One of the hallmarks of cancers is genetic instability, resulting in a variety of genetic aberrations, including aneuploidy, point mutations, deletions, gene amplifications, and chromosomal translocations (26). These result in altered gene and protein expression and substantial diversity among cellular populations in cancers. Goldie and Coldman formulated a mathematical model relating rates of generation of drug resistant mutations to the number of cells in a cancer at diagnosis, the probabilities for cure, and implications for various treatment strategies (27–29). Studies of human cancer cell lines using Luria-Delbrück fluctuation analysis have demonstrated mutation rates for drug resistance at a frequency of 10^{-6} to 10^{-7} per cell generation (30–33). Moreover, strategies which suppress one mechanism of resistance (activation of the *MDR1* or *ABCB1* gene) reduce the frequency of acquired resistance and result in the selection of alternative resistance mechanisms (32).

It is likely that the rate of development of drug-resistant mutants in a cancer varies depending on the nature of the genetic instability of that cancer, the drug mechanism, and the treatment dose and schedule (selection pressure; 27–29,34,35). In general, the concept of selection for acquired resistance implies that mutations may either be preexisting as small subpopulations within the cancer, or may arise during the course of therapy and eventually manifest themselves as regrowth of tumor.

Gene amplification, or increase in gene copy number, was first described for the *DHFR* gene as a mechanism for acquired resistance to methotrexate (36). Amplification of genes is now known to be a prominent feature of the genomic instability of cells,

and to be a key genetic mechanism involved in oncogenesis (*MYC*, *HER2*, *EGFR*), as well as in drug resistance. It is one of the major mechanisms for increasing the expression of drug resistance genes, including *MDR1/ABCB1* (26,37).

In addition to selection of resistant mutants, acquired resistance may develop via epigenetic changes, by induction of resistance gene expression. For example, various cellular stresses, including exposure to ionizing radiation and chemotherapies, have been shown to increase expression of the multidrug transporter gene *MDR1/ABCB1* (26,38–40).

Drug Efflux Transporters

There are approximately 50 ABC transporters (ATP-binding cassette membrane proteins) in the human genome (41–43). Defective forms of several of these transporters are causes of human genetic diseases, such as cystic fibrosis and Dubin-Johnson syndrome (41). Several members of the ABC transporter family have the capacity to efflux small molecules, including anticancer drugs, and thus contribute to drug resistance. The major drug-resistance ABC transporter genes include *MDR1/ABCB1*, several members of the *MRP/ABCC* subgroup, and *ABCG2* (43).

P-glycoprotein (P-gp), the product of the *MDR1/ABCB1* gene, is the most prevalent ABC drug-resistance transporter, and

has been extensively studied (44–48). The protein has a molecular mass of 180 kD, with 12 transmembrane segments and two intracytoplasmic ATP binding domains (Figure 49-2). High-resolution electron microscopy has revealed that the transmembrane segments form a pore, and drug binding sites have been identified within this pore. Access of drugs to the transporter is thought to occur both via the cytoplasm and by diffusion within the membrane. P-gp is a transporter with very broad substrate specificity, including approximately a third of all anticancer drugs, as well as many other drugs used in other areas of medicine. Active efflux of drugs is mediated by conversion of ATP to ADP. Theories regarding the molecular mechanism of drug extrusion include an ATPase-mediated conformational change in the protein producing a “flippase” action, which exposes substrate drugs to the extracellular environment, and a “membrane vacuum cleaner” function in which drugs access the transporter via the bilipid plasma membrane (45). The direct role of P-gp in conferring multidrug resistance has been confirmed by transfection of the gene in cellular models (47).

P-gp is expressed in many normal tissues, where it serves as a barrier to drug absorption (small bowel and colon), a barrier to tissue entry (endothelial cells of the CNS, testis, and placenta), and to facilitate drug excretion (biliary tract of the liver and proximal tubule of the kidney; 4,48). It is also highly expressed in cancers

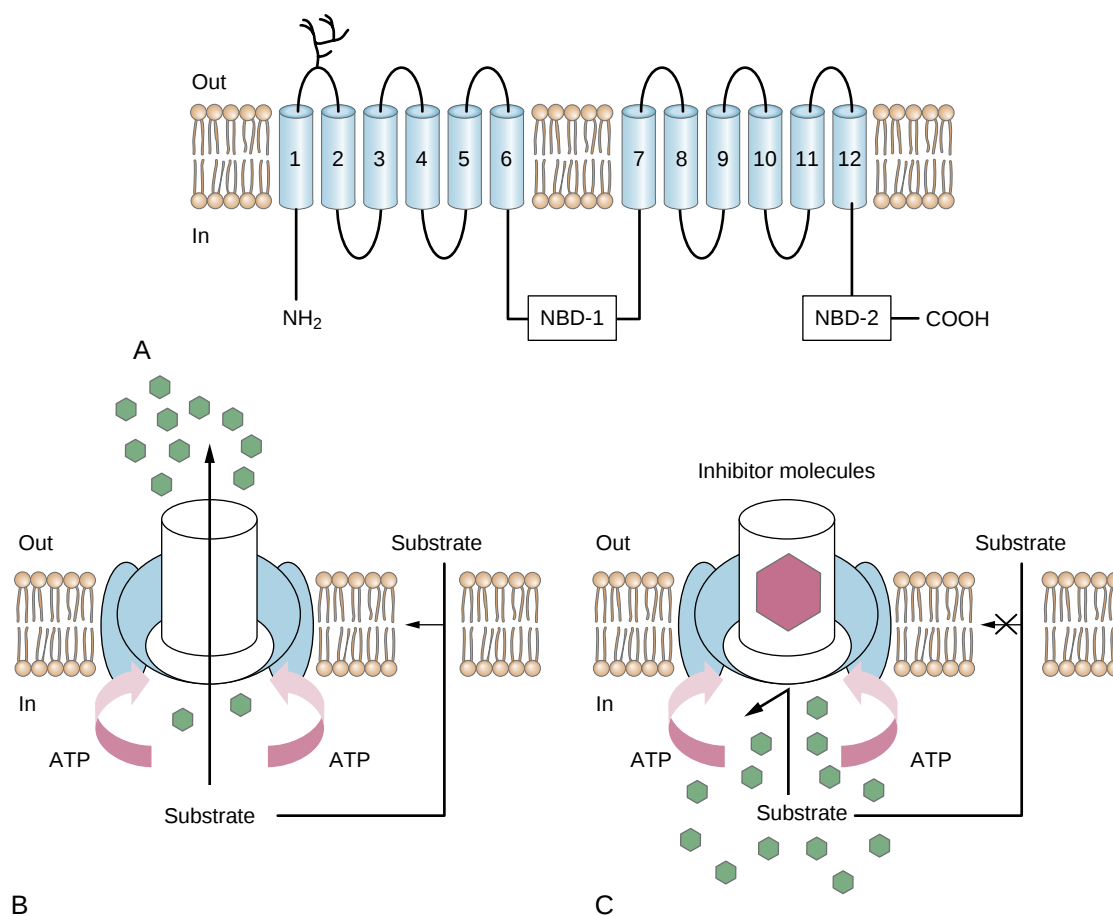


FIGURE 49-2 Structure and mechanism of action of P-glycoprotein (P-gp). **A:** Diagram of P-gp showing the 12 transmembrane segments, two nucleotide binding domains (NBDs) and extracellular glycosylation. **B:** P-gp forms a central pore and requires ATPase activity to pump drugs out of the cell. **C:** Inhibitors of P-gp function prevent drug efflux, resulting in increased intracellular drug accumulation and enhanced killing of multidrug-resistant cells.

derived from these tissues (colorectal, renal) and is one of the constitutive mechanisms of drug resistance in these cancers.

P-gp expression in cancers results in a classical, multidrug resistance phenotype, with high degrees of resistance to the drugs that are transport substrates for the protein. These drug substrates include the anthracyclines (doxorubicin, daunorubicin, idarubicin, and epirubicin), vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), mitoxantrone, and dactinomycin (45). Some newer, targeted drugs such as imatinib are also transport substrates for P-gp.

The clinical significance of P-gp in drug resistance is supported by evidence that its expression confers an adverse prognosis in many tumor types, including acute myeloid leukemias (AMLs), acute lymphoid leukemias, lymphomas, myeloma, breast and ovarian cancers, and sarcomas (8,49–55). In AML, P-gp is expressed in more than 70% of specimens from patients older than age 60, versus 30% to 40% of patients up to age 60, and its expression correlates with reduced rates of complete remission and shorter survival (54). In breast cancers, P-gp expression occurs in 40% to 50% of specimens and is associated with decreased rates of remission to P-gp substrate drugs (taxanes and anthracyclines; 55). Selection of multidrug-resistant (MDR) subclones within cancer populations is suggested by evidence that P-gp expression is more frequent in leukemias and breast cancers after patients have relapsed from prior therapy with MDR-related chemotherapy drugs (54,55).

The prevalence and adverse prognostic effects of P-gp in many cancers have led to attempts to reverse MDR by combining chemotherapy with inhibitors of P-gp (54,56–58). These clinical trials to reverse or modulate MDR have used a variety of competitive and noncompetitive inhibitors of P-gp, including verapamil, cyclosporine, quinine, the cyclosporine analog valsopodar, and others. In general, these attempts have not resulted in proven clinical benefit. The reasons for these failures are multiple, and include the following: inadequate concentrations of MDR-reversing agents because of toxicities to normal tissues, lack of specificity of P-gp inhibition leading to drug interactions and off-target effects, use of an unselected patient population including patients who did not express P-gp, and coexpression of other mechanisms of drug resistance (54,56–58). A particularly problematic issue is the co-inhibition by cyclosporins and other MDR inhibitors of other ABC transporters as well as the mixed-function oxidase CYP 3A4, resulting in the need to reduce doses of chemotherapeutic drugs while attempting to sensitize P-gp-expressing cancer cells (58–61). Despite these issues, cyclosporine has been shown to moderately increase complete remission rates and to significantly prolong survival in a randomized clinical trial in AML (54,62). A more potent and specific inhibitor of P-gp, zosuquidar, is currently being tested more extensively in this disease (63).

Several members of the MRP or ABCC gene family also function as drug transporters (64–72). The MDR-associated protein (the MRP1/ABCC1 gene) confers resistance to anthracyclines, vinca alkaloids, and epipodophyllotoxins and preferentially transports glutathione conjugates of substrate drugs (64–67,71,72). In general, MRP1 is not as strongly associated with clinical drug resistance and prognosis as MDR1, and clinical

strategies for reversing resistance related to MRP1 have not been developed. The MRP2/ABCC2 gene encodes the canalicular multiple organic anion transporter, which is expressed at high levels in the biliary tract, and transports glucuronide and glutathione conjugates of drugs, including anthracyclines. It plays a role in hepatic excretion of anticancer drugs, but its role in drug resistance is not clear (64,70). Its hereditary deficiency results in the Dubin-Johnson syndrome (68). The transporter encoded by the MRP3/ABCC3 gene confers low-level resistance to epipodophyllotoxins as well as to methotrexate (68,69). MRP4/ABCC4 and MRP5/ABCC5 confer resistance to anionic purines and other nucleotide analogs and their metabolites (68).

ABCG2 (BCRP) is another member of the ABC family, implicated in clinical resistance to the anthracenedione drug mitoxantrone and the camptothecins (73,74). This transporter is 72 kD in size, less than half the size of the ABCB and ABCC subgroups, and is thought to require dimerization for its function. It is variably expressed in AML and is a negative prognostic factor in that disease (75–77). Together with P-gp, ABCG2 has been shown to be constitutively expressed in both normal hematopoietic and leukemic stem cells (78,79).

Polymorphisms in the DNA sequence of ABCB1 and other ABC transporters are being studied for their relationship to drug disposition, efficacy, and toxicities (42). Single-nucleotide polymorphisms of the ABCB1 gene (C1236T, G2677T, and C3435T), which have been associated with altered drug absorption or disposition in some studies, were not found to affect complete remission and survival in patients with AML (80). The function and clinical significance of the ABC transporter family in anticancer drug resistance continue to be investigated.

Two membrane proteins involved in the efflux of copper, ATP7A and ATP7B, have been shown to also transport the platinum drugs, and contribute to resistance to cisplatin, carboplatin, and oxaliplatin (81,82).

Impaired Drug Uptake

Cellular entry of most anticancer agents is via passive diffusion. However, some drugs are also transported into cells by membrane proteins, and the expression and activity of these proteins are determinants of cellular sensitivity or resistance. Methotrexate enters cells by means of the reduced folate carrier, and decreased expression of this protein results in relative resistance to the drug (83). Reduced drug uptake has also been observed in some cells resistant to platinum drugs (84). The major copper influx transporter, CTR1, been implicated in the regulation of intracellular accumulation of cisplatin, carboplatin, and oxaliplatin (81).

Mutation or Altered Expression of Molecular Targets

As previously mentioned, the first description of gene amplification as a genetic phenomenon and as a mechanism for acquired drug resistance was the discovery of amplified dihydrofolate reductase (*DHFR*) genes in a cell line selected by exposure to increasing concentrations of methotrexate (36). Multiple copies of

DHFR were identified in extrachromosomal fragments of DNA, termed “double minute chromosomes” (DMs), in the methotrexate-resistant cells. Resistance in these cells was unstable because DMs were not normally replicated in the absence of drug selection (85). Subsequently, other methotrexate-resistant cells were found to have multiple gene copies of *DHFR* integrated into the genome, in areas of “homogeneously staining regions,” or HSRs. HSRs are more stable because they are integrated into the genome and included in the normal process of DNA replication.

Several important classes of anticancer drugs (vincas, taxanes, epothilones) act by binding to β tubulins and altering the dynamic instability of microtubules (Figure 49-3; 86). Alterations in β tubulins, including mutations and changes in the proportion of β -tubulin isoforms, particularly the class III isoform, have been implicated in resistance to taxanes (86–89). Vinca alkaloids inhibit tubulin polymerization, and thus have opposing effects to those of taxanes and epothilones, which stabilize polymerized microtubules. These opposing mechanisms of action may be reflected in reciprocal effects of changes in tubulin content or isotype expression on vinca and taxane sensitivities, with resistance to one class of drugs accompanied by increased sensitivity to the other (86). Although mutations in β -tubulin that alter taxane binding have been found to confer resistance in cellular models, such mutations have not been found in various human cancer clinical specimens (90,91).

The microtubule binding protein, MAP-Tau, binds to a site on β -tubulin overlapping with taxanes, and affects microtubule dynamic instability. Its expression has been associated with resistance to the taxane drug paclitaxel in breast cancer specimens (92,93). Other mechanisms of resistance to antitubulin drugs include the P-gp transporter (for taxanes and vincas; 55), the cell

spindle checkpoint control pathway (94), and regulation of programmed cell death or apoptosis (86,95).

The epothilones are a new class of antitubulin cytotoxic drugs whose binding site on β tubulins overlaps with the taxanes (96). In contrast to taxanes, epothilones are not transport substrates for P-gp, and therefore have potential antitumor efficacy in cancers which are multidrug resistant because of P-gp expression (96,97). However, they are likely to share some of the target-related mechanisms of resistance to taxanes, such as factors that affect microtubule dynamicity and regulation of apoptosis.

Topoisomerase I and II are drug targets for camptothecin and epipodophyllotoxin drugs, respectively, and mutations or altered expression of these enzymes have been shown to cause cellular resistance to these drugs (30,98–103). Because drug-induced DNA breakage is proportional to the amount of topoisomerase II enzyme, decreased enzyme content is associated with resistance, and higher enzyme content with drug sensitivity (30,99,102).

Alteration of drug targets is an important mechanism of resistance for new targeted drugs, such as the tyrosine kinase inhibitor imatinib (Figure 49-4). For this drug, point mutations in the kinase domain of its target, the fusion oncoprotein gene *BCR/ABL*, are a major mechanism of acquired resistance in chronic myeloid leukemias (CMLs; 104). More than 30 such mutations that confer resistance to imatinib have been identified. Since resistance to imatinib occurs at a rate of around 3% of patients per year of drug therapy, such mutations occur relatively infrequently. The drug dasatinib, a potent inhibitor of the *BCR/ABL* kinase, has been shown to inhibit almost all of these mutant kinases and to produce remissions in imatinib-resistant CML (104). One *BCR/ABL* mutant, T351I, remains resistant to both imatinib and dasatinib, although other new drugs are being developed for this double resistant mutation.

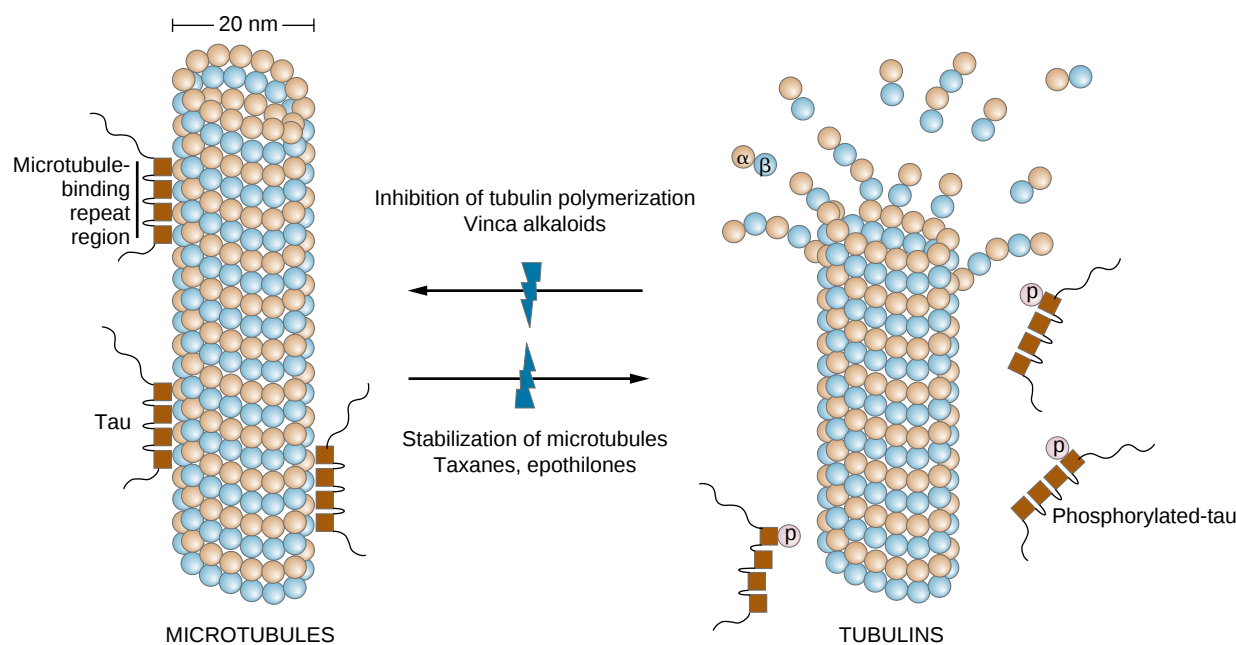


FIGURE 49-3 Mechanism of action of tubulin polymerizing and microtubule-stabilizing drugs.

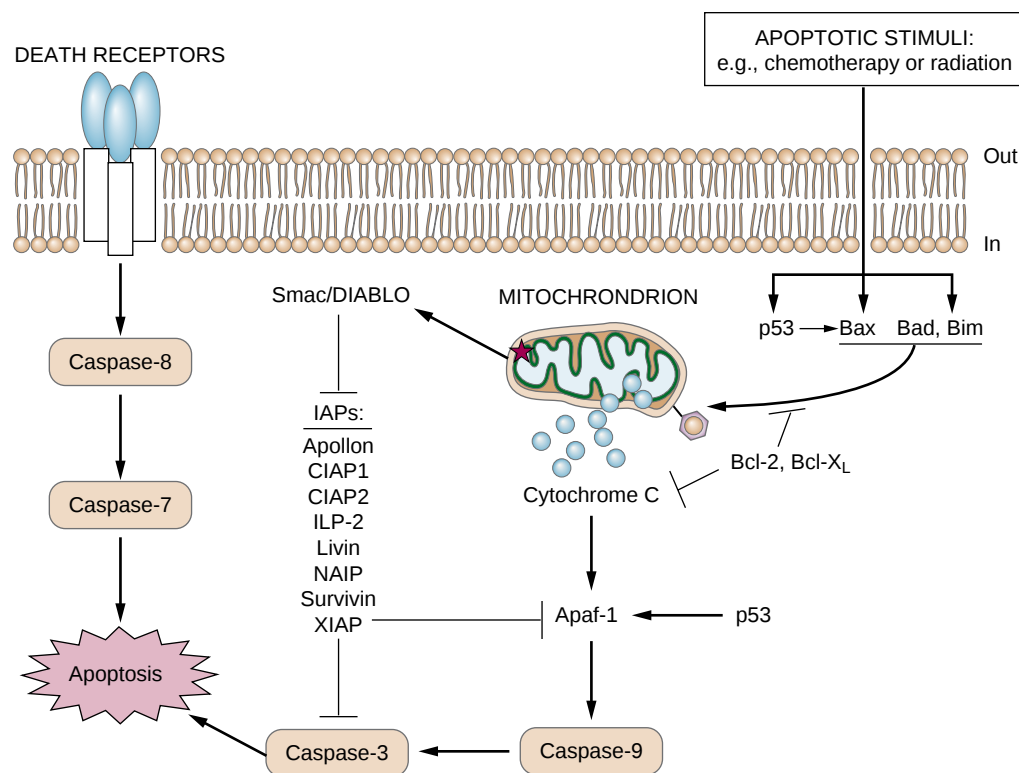


FIGURE 49-4 Cellular pathways of programmed cell death, or apoptosis.

Intracellular Redistribution of Drug

Intracellular drug sequestration of anthracyclines has been observed in cellular models with high expression of the major vault protein (MVP), also known as LRP (105). Vaults are barrel-like cytoplasmic organelles with a molecular mass of 13 MDa, which are thought to function in intracellular transport. In addition to high expression of MVP in some cellular models of drug resistance, this protein is variably expressed in acute myeloid leukemias, and may be a factor in clinical drug resistance in that disease (106).

Detoxification of Drug or Intermediate Drug Product

Metabolic inactivation of drugs is a mechanism of resistance to many agents. Thus, cytidine deaminase activity can result in resistance to cytarabine (107). Dihydropyrimidine dehydrogenase catabolism of 5-fluorouracil is a determinant of activity of that agent (108).

The DNA-binding glycopeptide drug bleomycin is inactivated by an aminopeptidase termed "bleomycin hydrolase" (109). Most cancers are resistant to bleomycin and have high levels of this enzyme, whereas sensitive tumors (germ cell cancers, lymphomas, squamous carcinomas) have low levels. Similarly, most normal tissues have high levels of bleomycin hydrolase, but the two major sites of toxicity, lung and skin, express low levels (109).

For electrophilic DNA alkylating agents and platinum drugs, detoxification via nucleophilic sulphur-containing compounds

is an important class of resistance pathways (82). Glutathione reductases are an important class of detoxifying enzymes that can generate resistance to such drugs by conjugation with glutathione (110–120). Moreover, as previously noted, some members of the MRP family of transporters can efflux glutathione conjugates of cytotoxic drugs, so that metabolic detoxification is coupled to outward transport of toxins (64,66,68).

Enhanced DNA Repair

DNA repair pathways are important determinants of response to alkylating agents and platinum drugs (82,121–124). Nucleotide excision repair (NER) is a complex, highly regulated process involving more than 30 proteins. Moreover, two general pathways are involved: global genomic NER, which repairs damage in transcriptionally silent areas, and transcription-coupled NER, which repairs damage to the actively transcribed DNA strand. The steps in NER include recognition of the damaged DNA, DNA unwinding, incision, degradation, polymerization, and ligation (123). Evidence for the role of many DNA repair genes in response to both DNA damaging drug and ionizing radiation derives in part from studies of genetic defects such as ataxia telangiectasia, xeroderma pigmentosum, and Bloom syndrome, in which hypersensitivity to DNA damaging agents has been observed.

Among the many genes involved in NER, recent attention has focused on *ERCC1*. High expression of the DNA excision repair gene, *ERCC1*, which is involved in repair of DNA adducts from alkylating agents and platinum drugs, has been shown to

correlate with adverse outcomes in patients with advanced-stage non-small cell lung cancers treated with cisplatin-based chemotherapy (125). In earlier stages of lung cancer, patients whose tumors did not express *ERCC1* benefited significantly from cisplatin adjuvant chemotherapy, whereas patients whose tumors expressed *ERCC1* did not benefit from the chemotherapy (126). Paradoxically, high expression of *ERCC1* was found to be a favorable prognostic factor for survival in patients with early stages of lung cancer, in the absence of adjuvant chemotherapy (126–128).

O⁶-Methylguanyl-methyl-transferase (MGMT) is particularly important in resistance to the nitrosourea carmustine and the DNA-methylating agent temozolomide (121,123). MGMT has been identified as a key factor in clinical outcomes in brain tumors, and drugs to deplete MGMT are being developed as potential therapeutic approaches to modulate drug resistance (121).

Decreased Drug Activation

Most antimetabolite drugs generally require metabolic activation to generate their active nucleoside or nucleotide moiety, via kinases and phosphoribosyl transferases (129). Thus, for cytarabine, generation of ara-dCTP levels intracellularly is an important determinant of antitumor efficacy (129). In the case of 5-fluorouracil, activation of the drug requires formation of 5-fluorodeoxyuridine mono-phosphate (FdUMP; 129). In addition, optimal inhibition of thymidylate synthase by 5-fluorouracil depends in part on intracellular levels of the cofactor 5, 10-methylene tetrahydrofolate (130).

The oxazaphosphorine mustards (cyclophosphamide and ifosfamide) are prodrugs that are activated predominantly in liver tissue by mixed function oxidases (CYP enzymes; 122). Although the major mechanisms of resistance to these drugs are thought to be inactivation of alkylating metabolites by thiol compounds, as well as DNA repair mechanisms, variable levels of mixed function oxidase activity within cancers may also be a determinant of their activity.

Altered Pathways for Programmed Cell Death (Apoptosis)

Pathways for the regulation of programmed cell death or apoptosis are important both in oncogenesis and as determinants of response to cancer therapies (Figure 49-5; 11–24). *BCL2* is oncogenic in many B-cell lymphomas, where its expression is up-regulated by chromosomal translocations and other mechanisms. It also functions to protect cells from apoptosis after radiation, glucocorticoids, and chemotherapies (13,22,23). The *BCLX* gene has long and short forms, encoding the proteins bcl-xL and bcl-xS, which serve to inhibit and promote apoptosis, respectively (11).

The relationship between oncogenesis and drug sensitivity or resistance is also exemplified by the p53 pathway, which is mutated in the majority of human cancers. Normal p53 function is essential for the efficient functioning of the mitochondrial mediated apoptotic pathway, particularly in response to DNA damaging agents, including ionizing radiation and many chemotherapeutic drugs, such as alkylating agents, platinum, anthracyclines, and topoisomerase inhibitors (12,14,21,24).

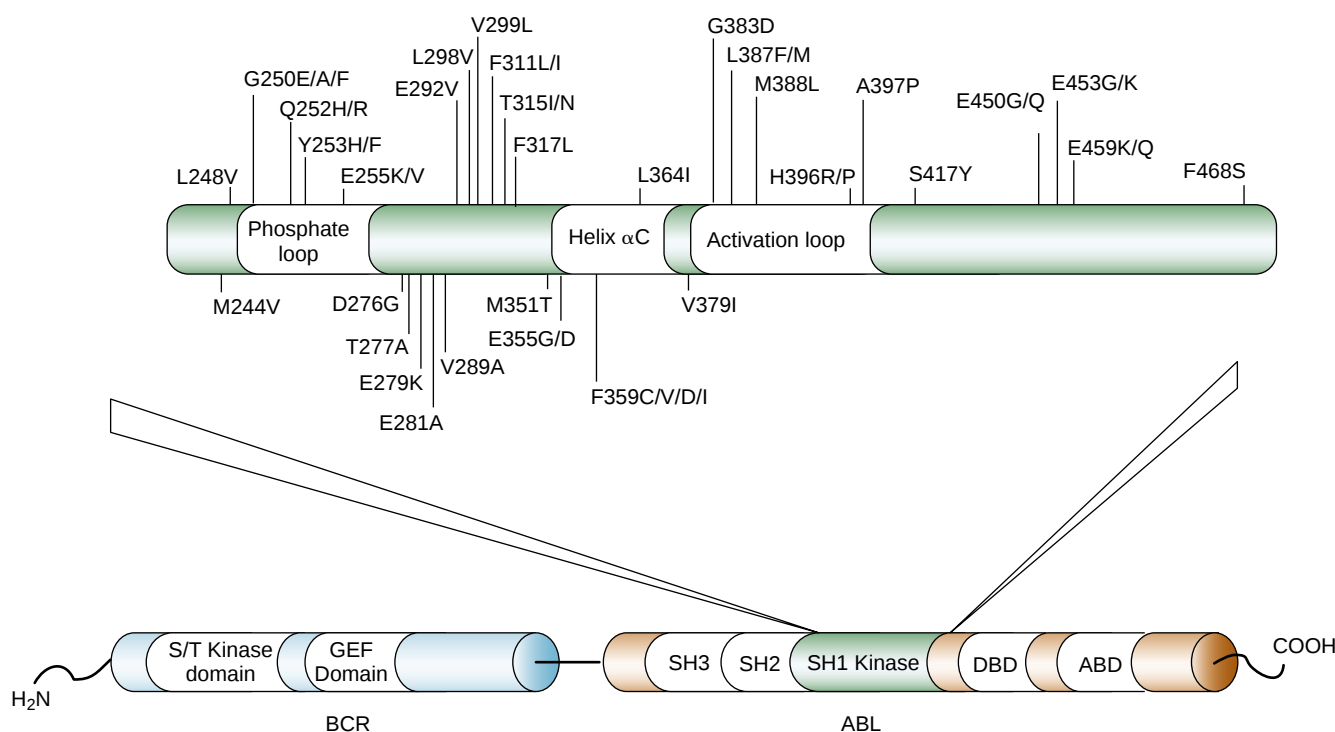


FIGURE 49-5 Structure of the fusion oncoprotein BCR/ABL, depicting the point mutations, which result in resistance to imatinib in chronic myeloid leukemias.

Individualization of Therapy Based On Predictive Multigenic Markers

Knowledge about mechanisms of drug resistance, molecular targets of drugs, and signaling pathways related to treatments are enabling more precise predictive molecular testing of drug efficacy (9). Historically, such approaches were pioneered in the treatment of breast cancer, by the use of hormone

receptor measurements to guide hormonal therapy, and testing for overexpression or amplification of the *HER2* gene to identify breast cancer patients for trastuzumab therapy. The ability to determine genome-wide expression by microarray analysis has resulted in the identification of candidate gene profiles that are associated with remissions to drugs or drug combinations (131,132). Such approaches will lead to increasing individualization of therapy with the use of genomic or proteomic panels of predictive markers.

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Molecular Basis of Radiation Therapy

As a critical component of multidisciplinary cancer care, radiation therapy is used in the treatment of over 50% of patients with cancers of a variety of organ systems. Radiation has several important benefits. Radiation has been shown to improve overall survival alone and in combination with chemotherapy by improving local tumor control (see [1] for review). Use of radiation provides an organ-sparing approach for many patients with early-stage cancer (1), and it is effective for symptom palliation from metastatic disease (2). Radiation therapy has witnessed numerous clinical advances in recent decades as the result of an increasing understanding of the biology of the interaction of radiation with normal and tumor tissue. This understanding continues to guide ongoing clinical trials, including radiosensitization with novel targeted therapies and normal tissue protection.

Classic radiobiology groups radiation-mammalian cell interactions into four major categories, known as the four R's of radiobiology: repair, redistribution, reoxygenation, and repopulation. On the down side, the tumor uses DNA repair by tumor cells after radiation (Figure 50-1) and repopulation of the tumor, presumably by resistant clonogens in response to radiation or other environmental stresses (Figure 50-2), to escape cell death. Conversely, tumor cell kill by radiation is improved through the redistribution of tumor cells, such that a greater proportion is in a more radiosensitive stage of the cell cycle (Figure 50-3), and through the reoxygenation of previously hypoxic and therefore radioresistant cells (Figure 50-4). In this chapter, we discuss the main elements of classic radiobiology as well as the molecular signaling that produces these effects. Because of the importance of molecular signaling, we propose a fifth 'R' of radiobiology: molecular regulation (R_m). This is because genomic, message, and proteomic effectors in both tumor and stromal cells clearly regulate the other four R's (Figure 50-5). New technologies have led to the interrogation of global gene expression and subsequent protein regulation in response to radiation and are providing novel insights into the mechanisms of radiation response and sensitivity. For example, gene expression profiling done in preclinical and clinical tumor samples has identified profiles that can predict response to chemoradiation therapy (rectal cancer), resistance to chemoradiation therapy (high-risk head and neck cancer), and site-specific distant metastasis (4–6). Comparisons of low- and

high-dose radiation exposure in both tumor cells and stromal fibroblasts have demonstrated unique, dose-dependent signaling responses within cell lines (7,8). Array studies have demonstrated markedly different *p53* signaling in cells from different tissues irradiated with the same energy (9,10) and have shown that distinct tissues that exhibit the same radiation-induced functional response may do so through differing transcriptional mechanisms (11). Such global genomic comparisons are not only elucidating the pathways involved in the radiation response but also highlighting the complexity and heterogeneity of these responses and the importance of considering response to radiation in the context of the microenvironment and as a part of a complex three-dimensional system.

Radiation-Induced Cell Death

DNA as the Target

The most well-studied target of radiation-induced cell death is DNA. This is because, although all components of the cell can theoretically be damaged by radiation, studies in which the cytosolic and nuclear compartments were radiated independently demonstrated that the main target of *direct* radiation damage is the nucleus. Indirect damage to cells adjacent to radiated cells may be effected by other mechanisms, as will be discussed later. Fluorescent antibodies directed toward proteins in the DNA-damage surveillance complex (i.e., ATM and γ -H2AX) have allowed the kinetics and stoichiometry of DNA damage to be elucidated; these studies also provided new tools for investigating radiation resistance and repair in single cells (Figure 50-6; 12,13). Mutations of the ATM protein kinase are associated with ataxia telangiectasia, a disorder characterized by extreme sensitivity to radiation-induced DNA damage. Bakkenist et al. (12) showed that ATM is held in unirradiated cells in the form of a dimer or higher order multimer with the kinase domain bound to a region surrounding serine1981. Cellular irradiation as low as 0.5 Gy leads to autophosphorylation of this site and dimer dissociation that initiates ATM kinase activity, which is detectable by specific antibodies to ATM serine1981 after only a few DNA double-strand breaks.

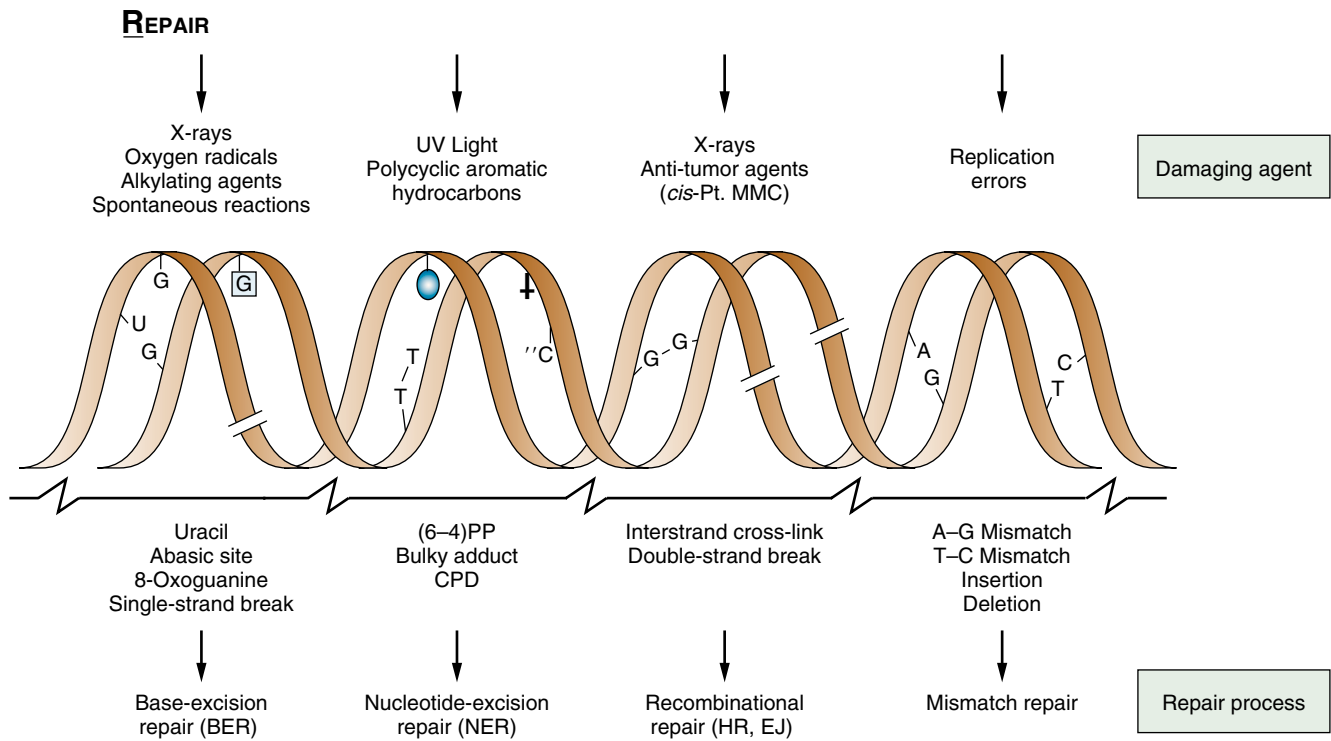
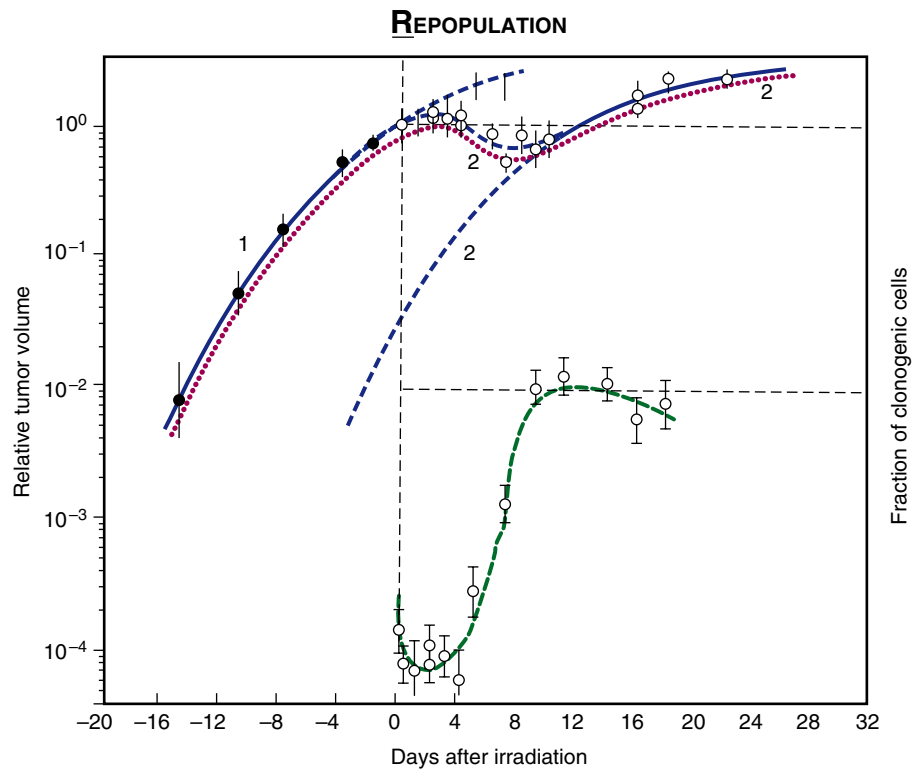


FIGURE 50-1 Repair: Radiation-induced DNA damage starts to be repaired within minutes by error-prone and error-free processes. X-rays, the kind of radiation most often used in the treatment of cancer, can cause several types of DNA lesions including single- and double-strand breaks. Failure to repair double-strand breaks ultimately results in mitotic catastrophe and cell death. Different types of damage are repaired by different mechanisms including base excision repair, nucleotide excision repair, homologous recombination (HR, error-free) and nonhomologous end joining (EJ, error-prone). Molecular regulators of repair are distinct for each type of repair process. Key examples include ATM, Rad51, BRCA, Ku proteins, DNA-PK, and γ -phospho-H2AX. (From Ref. 3.)

FIGURE 50-2 Repopulation: Tumors are heterogeneous populations of cells with differing radiation sensitivity independent of the cell cycle. When a sufficient dose is given in a single fraction, tumor regression can be clinically appreciated. This is depicted by the red dotted line (tumor volume) that increases until time zero when radiation is given and decreases for about 12 days after radiation. However, even while tumors are regressing, the proportion of *clonogenic cells* within the tumor (i.e., cells capable of indefinite replication and therefore potentially tumor recurrence) may increase as less clonogenic, more radiosensitive cells are selected against and killed. In the green bottom curve, the fraction of clonogenic cells within the tumor after irradiation is depicted. Although the tumor is grossly shrinking (shown by the flattening of the red line), the bottom curve demonstrates clonogenic repopulation of the tumor is accelerated with time after radiation, and that the normally small clonogenic fraction of the tumor volume increased by two logs before the tumor started to grow again (shown by the upward trending red line 12 days after radiation). In the clinic, the interval between radiation fractions is sometimes shortened to compensate for this phenomenon. Molecular regulators of stem cell survival/self-renewal such as Wnt, Notch, Sonic Hedgehog and Bmi have been proposed as regulators of this phenomenon. (From Hall EJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Wilkins, 2000.)



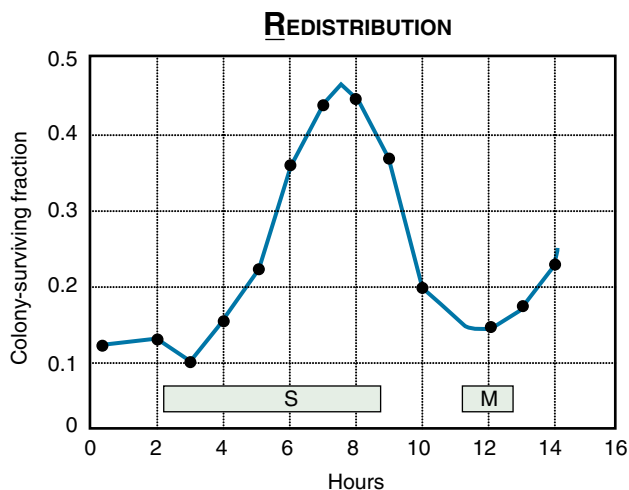


FIGURE 50-3 Redistribution: In an unsynchronized population of cells as tumors are, cells in late S-phase are most resistant to radiation. After radiation however, cells accumulate in the G_2/M phase, when they are most sensitive to radiation. Therefore cells that were not lethally damaged after one dose of radiation may cycle and redistribute themselves into the more sensitive G_2/M phase after radiation and be more susceptible to the next dose. In the graph, cells were synchronized to enter the cell cycle together. At two to eight hours after synchronization all cells are in S phase. By 12 hours they are in G_2/M . Colony-surviving fraction, a measure of resistance, is highest in the cells in late S-phase. Key proteins involved in cell cycle regulation include the cyclins, cdk, p53 and p21. (From Hall EJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Wilkins, 2000.)

Radiation damage can cause both single- and double-strand DNA breaks, and multiple pathways are involved in mediating the cellular response to ionizing radiation. Nonetheless, they culminate in either cell checkpoint arrest, which allows the cell to undergo repair, or the activation of cell death pathways through necrosis or apoptosis. Cells with cell cycle checkpoints activated through a damage surveillance complex (RAD, BRCA, NBS1) respond via a signal transduction mediated by ATM and CHK, which regulates downstream cell cycle regulators such as p53, p21, and CDK (see [14] for review). These cells accumulate in G_2/M and undergo repair. Single-strand breaks are generally repaired by nucleotide excision repair, a relatively error-free process that allows

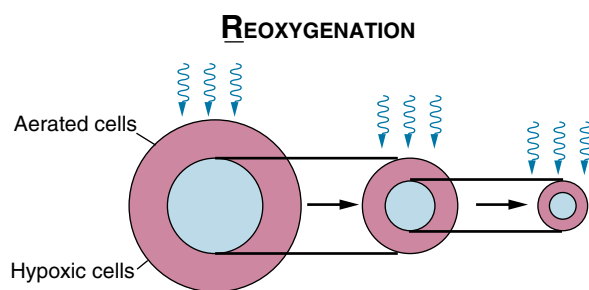


FIGURE 50-4 Reoxygenation: Hypoxic cells are less sensitive to radiation. Oxygen is thought to make permanent the damage caused by free radicals, thereby making the cells more sensitive to radiation. By killing the outermost layer of cells that are closest to the blood supply and therefore the best oxygenated, the inner cells become closer to the blood supply and become more sensitive to the next fraction of radiation. This process is repeated after each fraction of radiation. Candidate molecular regulators involved in this process include HIF-1 α , VEGF, NO, and bFGF. (From Hall EJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Wilkins, 2000.)

cells to successfully pass through G_2/M . Double-strand breaks are repaired by one of two mechanisms: nonhomologous end joining, an error-prone ligase-mediated process that rejoins the break such that a portion of a chromosome is not fatally lost during mitosis, and homologous recombination, a more complex, error-free process that requires the alignment of sister chromatids to repair the break. Cells are therefore most sensitive to radiation during the G_2/M phase, as a double-strand break at this time will likely lead to aberrant chromosome formation during mitosis and eventual cell death. Radiation delivered in multiple fractions takes advantage of G_2/M sensitivity, since the interval between fractions allows time for more resistant, non- G_2/M cells not killed in response to the first fraction to progress to G_2/M before the next fraction.

Since chromatin is most condensed during the G_2/M phase, it has been suggested that radiosensitivity is mediated in part by the chromatin structure. This is because, theoretically, a photon traveling through condensed chromatin passes through more DNA, which is more likely to cause an irreparable double-strand break. To verify this, Chapman et al. (16,17) used scanning electron microscopy to quantitate chromatin density and found in multiple cell lines that chromatin compaction indeed correlated with increased radiation sensitivity. These authors also demonstrated that chemicals that induce chromatin compaction, as indicated by increased H-3 phosphorylation, can induce radiosensitization. This work has important implications for the use of histone deacetylase inhibitors, which can increase chromatin compaction as well as interact with DNA repair pathways. These inhibitors are in clinical trials. Interestingly, numerous studies have now demonstrated their effectiveness as radiosensitizers in tumor cells and radioprotectors in normal tissue (see [18] for review).

The dogma that DNA damage in target cells is the primary mechanism of radiation-induced cell killing was recently challenged, however. In particular, Paris et al. (19,20) suggested on the basis of observations in endothelial cells of the intestine that there is a massive wave of apoptosis in response to large-fraction irradiation that is responsible for the death of stem cells in the crypts and that this eventuates in the gastrointestinal syndrome. They further showed that endothelial cell apoptosis in response to radiation is abrogated in acid sphingomyelinase knock-out mice and in mice treated with intravenous basic fibroblast growth factor, an endothelial cell mitogen and survival factor. Although this effect was observed only after a single, large dose of radiation rather than after more clinically relevant fractionated radiation, this offers a provocative new perspective on the biology of radiation-induced cell death consistent with a two-compartment model for intestinal cell killing (21) and further emphasizes the validity of multisystem tissue interactions in radiation response (22).

Mechanisms of Radiation-Induced Cell Death: The Role of Long-Term Clonogenic Assays

The relevance of apoptosis as the mechanism of cell death after radiation, and to some degree any cancer therapy, is now a matter of some debate (see [23] for review). The hematopoietic and lymphoid tissues appear to be sensitive to cell death caused by

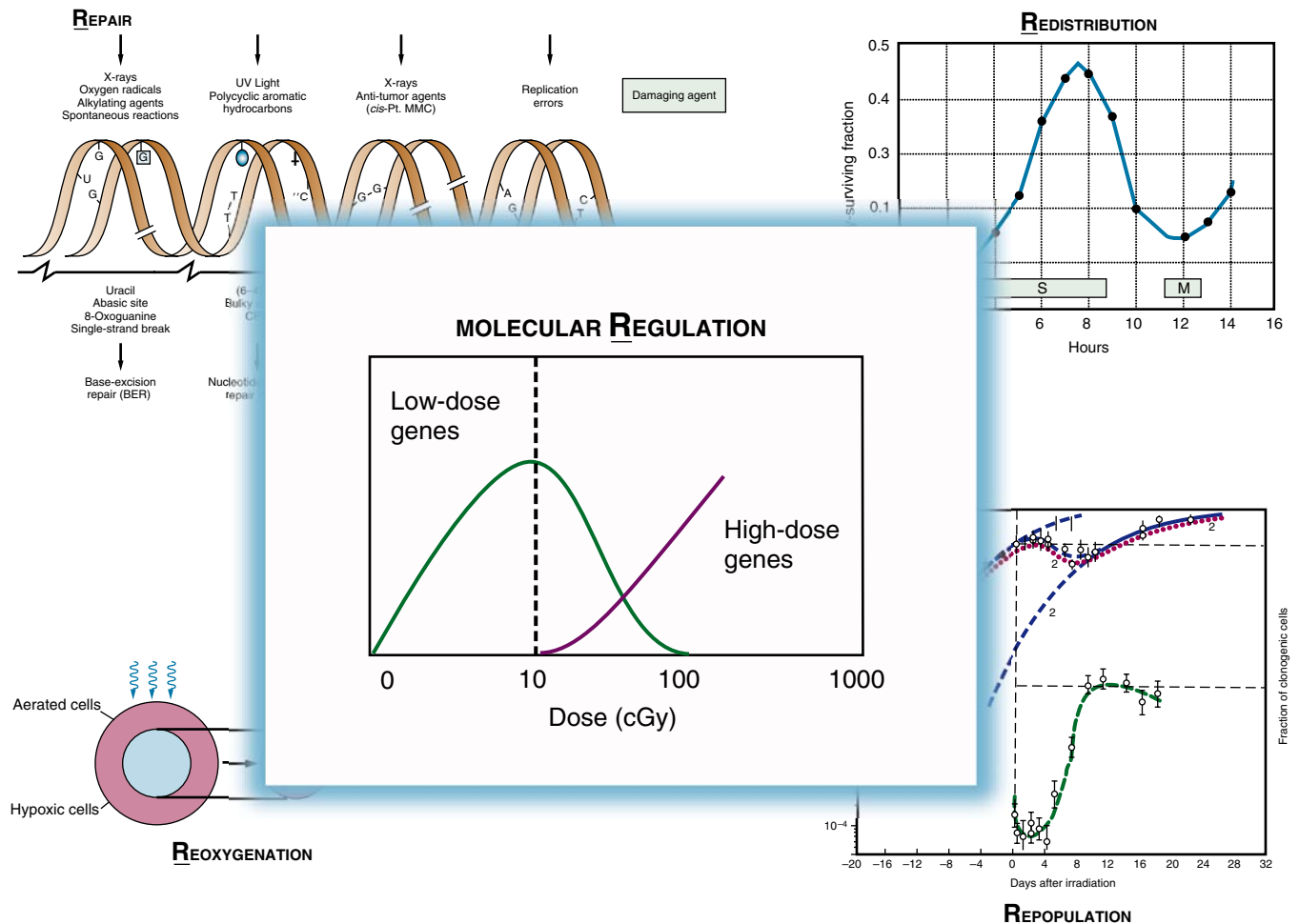


FIGURE 50-5 FIVE R'S OF RADIOBIOLOGY. Classic radiobiology teaching includes “the four R’s” of radiobiology: Repair, Redistribution, Reoxygenation, and Repopulation. Herein we propose a fifth R, molecular Regulation (R_m) to merge the detailed understanding of the biophysical aspects of the 4 R’s of radiation biology with the ever-expanding understanding of the molecular mechanisms integral to these four principles. **Molecular Regulation (R_m):** Molecular regulation controls all four mechanisms of radiobiology. This involves complex signaling processes that differ by cell type, cell context, radiation dose, and radiation energy that govern the cellular response to radiation. A representative depiction of the impact of dose on gene expression is depicted in the center. Other molecular changes induced by radiation include genomic stability, chromatin silencing, protein expression and function, and up or down-regulation of signaling pathways. These pathways also constitute targets for newly emerging targeted therapies that have radiosensitization and radioprotection effects. (From Hall EJ. *Radiobiology for the Radiologist*. Philadelphia: Lippincott Williams & Wilkins. Based on data from Yin E. *Int J Radiat Biol* 2003;79:759. Adapted by Anton L. Brooks.)

apoptosis (23), and radiation rapidly activates the apoptosis cascade, causing death by apoptosis within 4 hours after exposure. However, this does not appear to be the case in cells of epithelial origin. Although apoptosis can be induced in these cells by massive doses of radiation, the apoptosis that occurs more than 24 hours after exposure to clinically relevant doses of radiation is more likely to be a secondary effect of mitotic cell death.

Short-term assays of radiation sensitivity are not always appropriate for measuring cell survival because it can take as many as nine mitoses before moderate damage leads to cell death. This was corroborated in a comparison of short-term and long-term cell death assays using mouse embryo fibroblasts (MEFs) from wild-type and knock-out p53 mice. In this study, Brown and Wouters (see [23] for review) demonstrated a significant reduction in the viability of MEFs from p53 homozygous mice as opposed to p53 null mice after etoposide treatment, whereas clonogenic survival was identical in both cell populations. Zamble et al. (see Ref. 23 for review) presented similar data regarding p53-induced apoptosis.

Long-term clonogenic assays are a critical component in evaluating the effectiveness of radiation therapy and radiation sensitizers.

The preference for using long-term clonogenic survival assays in the field of radiobiology has meant that much insight has been gained into the re-emerging field of cancer stem cells. Although all clonogenic cells may not be stem cells, all stem cells are likely clonogenic, and there are direct correlations between clonogenic plating efficiency, transplant efficiency, and tumor control dose (24). Clonogenic cells are capable of iterative cell division after radiation treatment, as demonstrated in colony-forming assays performed on plastic, agar, or matrix implying these cells could mediate recurrence if not killed. True stem cells from a particular organ or cancer have this property of long replicative potential, as well as the ability to divide asymmetrically to both self-renew and differentiate into the cellular components of that organ or tumor.

The development of a simple, reproducible in vivo clonogenic assay in the jejunum whereby the effects of radiation on stem cells can be easily measured by counting crypts has been the

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FIGURE 50-6 Distributions of DNA damage foci over time after focused, alpha radiation (here stained pink using anti- γ -phospho-H2AX antibody and immunofluorescent detection) in HeLa nuclei at (A) 2.5 minutes, (B) 7.5 minutes, (C) 15 minutes, and (D) 60 minutes after irradiation. DNA repair antibodies now allow for quantitative visualization of DNA double-strand breaks after radiation. This is an important tool for examining and quantifying DNA damage in differing cell types under differing conditions. Radiation caused double-strand breaks, which are effectively marked by the γ -H2AX protein during the process of repair. These foci resolve after DNA damage repair is complete. (From Aten JA, Stap J, Krawczyk PM, et al. Dynamics of DNA double-strand breaks revealed by clustering of damaged chromosome domains. *Science* 2004;303:92–95.)

source for several decades of radiobiologic data demonstrating the response of normal stem cells to radiation. The conclusions that can be drawn from these data are that, in the small intestine, the cells at position 4–5 in the normal crypt, now identified as the stem cells, are exquisitely sensitive to radiation and undergo p53-dependent apoptosis within 2 to 3 hours after treatment with 1 Gy of radiation. However, a second population of *potential* stem cells exists that can be called into action in the event of lethal damage to the stem cells. That is, at low doses of radiation, the crypt of the small intestine contains four to five clonogenic regenerating cells, but at higher doses of radiation, up to 30 to 40 potential clonogenic regenerators can be called into play (25).

Evidence for the existence of *cancer* stem cells, a limited population of tumor cells that gives rise to all components of a heterogeneous tumor, was first obtained in acute myelogenous leukemia. It was shown in this context that only a minority of the leukemic cells had the pluripotency necessary to reconstitute tumors in the bone marrow of NOD/SCID mice (26,27). Subsequently, in a landmark study published in 2003, Park et al. (28) provided proof of principle that inhibiting the tumor stem cells could prevent recurrence of leukemia. Until recently, however, the prospective identification of *solid tumor stem cells* has remained elusive. However, Al-Hajj et al. (29) have now isolated a subpopulation of highly tumorigenic breast cancer cells from the bulk of human breast tumor cells using the cell-surface phenotype $\text{lin}^- \text{CD44}^+ \text{CD24}^-$, and prospective tumor stem cell markers have now also been identified in pediatric and adult central nervous system tumors (30). In normal mammary gland cells, markers (i.e., $\text{lin}^- \text{CD24}^+ \text{CD29}^+$) that identify single cells from which the entire gland can be regenerated have also recently been reported (31).

To date, there are limited data explicitly examining the link between clonogenic cells and stem cells. In the normal mammary gland Stingl et al. (32) describe the phenotype of the mammary gland regenerating unit as $\text{lin}^- \text{CD24}^{\text{hi}} \text{CD49f}^{\text{hi}}$

and report that the colony-forming or clonogenic cells have the $\text{lin}^- \text{CD24}^{\text{hi}} \text{CD49f}^+$ phenotype, but CD49f expression is low (CD49f^{lo}) rather than high in these clonogenic cells. These data thus support the hypothesis that stem cells and clonogenic cells are not identical and that historical clonogenic data may be more applicable to progenitor cells than to true stem cells. Several recent studies suggest that prospectively identified progenitors are relatively radiation resistant compared to more differentiated cancer cells (33–36), and that radiation resistance in these cells may be mediated by known stem cell self-renewal pathways such as the Wnt/ β -catenin pathway (Figure 50-7). These data suggest that targeting molecular pathways involved in stem cell self-renewal may be a new strategy to sensitize progenitors to radiation.

Molecular Regulation of Radiation Response

Cytokines, Growth Factors, and Signaling Pathways

Numerous ligands and growth factors have been described that stimulate or inhibit cell signaling pathways and mediate the effects of radiation in normal and tumor tissue. Understanding the effects of radiation on growth factors and signaling pathways has been difficult at times because of conflicting data that in fact reflect the enormous complexity and heterogeneity of signaling pathways that are present in different cells and different tissues depending on the timing and dose delivered as well as the expression of additional growth factors and the presence of mutations such as those that affect p53. Therefore, demonstrating that ionizing radiation acts on a particular growth factor to induce a particular signaling pathway in one cell does not necessarily mean that the same pathway is

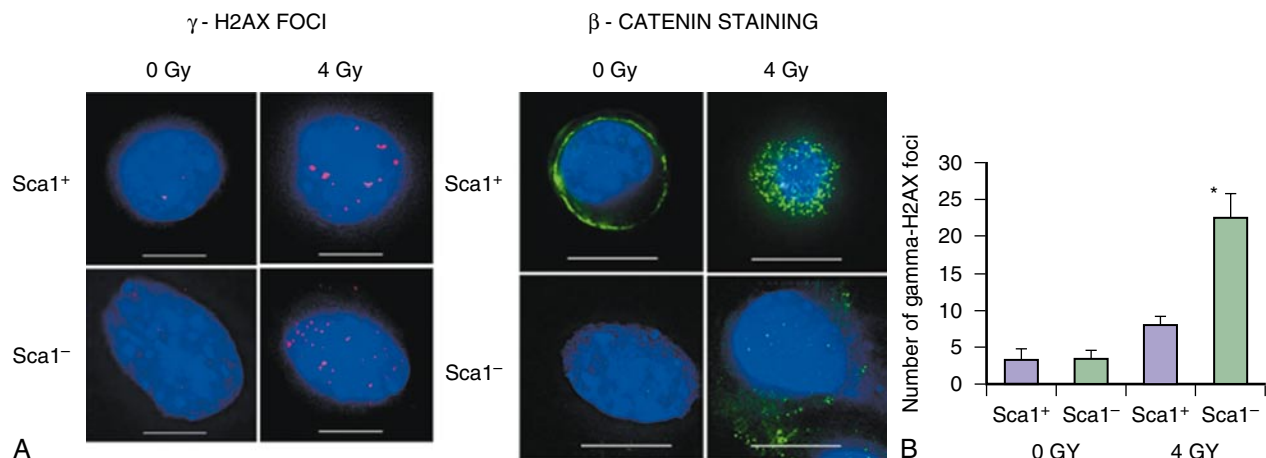


FIGURE 50-7 Radiation resistance of progenitors is associated with altered DNA damage response and increased β -catenin signaling. Murine mammary gland comma-D cells were irradiated and sorted 2 hours later using flow cytometry into progenitor (Sca1⁺) and nonprogenitor (Sca1⁻) fractions based on the expression of the progenitor marker stem cell antigen 1 (Sca1). DNA double-strand breaks were stained with γ -phospho-H2AX (A-red, left) and quantified (B). Fewer double-strand breaks were seen 2 hours after radiation in progenitor (Sca1⁺) cells compared with nonprogenitors (Sca1⁻). Cells were independently stained for activated (unphosphorylated) β -catenin (A-green, right). Increased β -catenin staining is seen in progenitor cells, and β -catenin localization is distinctly altered after radiation in progenitor cells. Concomitantly, downstream β -catenin effector survivin is differentially up-regulated in progenitor cells after irradiation, data not shown. (Adapted from Chen MS, Woodward WA, Behbod F, et al. *Wnt/beta-catenin mediates radiation resistance of Sca1+ progenitors in an immortalized mammary gland cell line*. *J Cell Sci* 2007;120(Pt 3):468–477, with permission.)

activated in another cell (see [37] for review). Indeed, potentially through the generation of reactive oxygen and nitrogen species that can inhibit protein tyrosine kinase phosphatase activity, ionizing radiation has been shown to activate multiple signaling pathways, including MAPK, JNK, PI3K, FAS-R, and TNF-R, that in turn affect cell survival and mitogenic responses, including anti-apoptosis effects and NF- κ B-mediated transcription. Further, while, for example, signaling through the PI3K pathway via EGFR1 appears to have a cytoprotective effect in all systems, the response to MAPK inhibitors varies significantly from system to system in response to radiation [37]. Further highlighting the complexity of this issue, additional cytokines such as tumor necrosis factor- α , interleukin-6, and transforming growth factor- β (TGF- β) have all been put forth as mediators of radiation-induced cell signaling [38–43]. Of these, TGF- β is perhaps the most extensively studied.

TGF- β represents a family of cellular mediators and consists of three isoforms, β 1, β 2, and β 3, that are essential for cell survival (see [97,98] for reviews). TGF- β signaling is mediated by two TGF- β receptors and transduced by the Smad family of signaling molecules that can act as transcription factors when activated [44,45]. TGF- β 1 has three main functional roles in vivo: general inhibition of cell growth, immunosuppression, and regulation of the extracellular matrix (ECM), but these roles vary depending on the tissue type and context (see [46] for review). Accordingly, the dysregulation of TGF- β in response to radiation has been linked to carcinogenesis (see [47] for review), radiation-induced fibrosis in normal tissues (see [48] for review), the growth arrest and differentiation of nontransformed cells [49], and cell protection or apoptosis in tumor cells depending on the cell type [50]. The effect of radiation on the immune function of TGF- β is less well studied. In this regard, Mason et al. [51,52] used peritumoral injections of CpG DNA oligonucleotides, a powerful agonist of the toll-like receptor-9, to stimulate the immune system and studied the effect of this in combination with radiation administration. These researchers observed dramatic antitumor responses to the combination.

It is unclear why CpG administration alone has proven insufficient to induce an anti-tumor response; however, Chow et al. [53] reported their finding that TGF- β may be required for CpG-specific signal transduction, which may indicate that TGF- β is involved in mediating the effects of this combination on the immune system.

Rapid increase in TGF- β 1 expression in response to radiation has been described in virtually every tissue that develops fibrosis in response to radiation (see [48] for review). Recent advances in our understanding of the molecular mechanisms of fibrosis have now recast fibrosis as being at one end of the spectrum of wound healing that may not be irreversible, as previously believed. The process by which normal cells become fibrotic has been termed “epithelial-mesenchymal transition” and depends on the balance between fibrosis induction mediated by TGF- β 1 and fibrosis inhibition mediated by the bone morphogenic protein-7 (BMP-7; [54]. Lin et al. [55,56] further demonstrated that kielin/chordin-like protein facilitates the action of BMP-7 by trapping the ligand to its receptor and suppressing TGF- β signaling, which negatively regulates fibrosis in the kidney. These data underscore ongoing clinical efforts to reverse radiation-induced fibrosis with negative regulators. Liposomal Cu/Zn superoxide dismutase (SOD) is the first agent shown to be effective in reducing long-standing fibrosis in patients treated with radiotherapy [57]. This effect is thought to be secondary to an SOD-mediated reduction in TGF- β 1 and its downstream target tissue inhibitor of metalloproteinase [58]. In pig models, the co-administration of pentoxifylline and α -tocopherol (vitamin E) were found to markedly reduce subcutaneous fibrosis [59,60], although pentoxifylline alone has only been used successfully to heal radiation necrosis [61,62].

Environmental Interactions

Signaling and interactions between cells also govern the molecular aspects of radiation response. For example, experimental studies

have shown that the genomic effects of radiation are evident in unirradiated cells adjacent to cells damaged by radiation, a result known as the bystander effect (63), as well as in the unirradiated progeny of irradiated cells (64). Gene expression analysis of unirradiated cells treated with media from irradiated cells has demonstrated the upregulation of 15 genes involved in cell-cell communication (65) and has shown that this up-regulation can be mediated by soluble, secreted effectors (see [66–68] for review). Similar studies of bystander cells exposed to the media of irradiated cells have demonstrated that irradiated media leads to increased levels of activated JNK and ERK proteins and that while the inhibition of JNK decreases bystander-induced apoptosis, the inhibition of ERK increases it (69). The bystander effect can also be mediated by direct junctional communication between cells and, in this case, is predominantly attributed to changes in connexin 43 (see [63] for review). Indeed, the bystander effect is not seen in connexin 43-deficient cells in a three-dimensional model system (70). Although the processes involved in the repair of double-strand breaks have been implicated in the mechanism of the bystander effect (71,72), targeted microbeam irradiation of specific parts of the cell has shown that DNA damage is not required to elicit the bystander effect (73), further challenging the dogma that DNA double-strand breaks are the primary target of radiation.

To shed light on the signaling pathways responsible for the bystander effect, Zhou et al. (74) compared gene expression in irradiated and bystander normal human lung fibroblasts using signal transduction specific gene arrays and found that only one of the 96 genes in the array, cyclo-oxygenase 2, was consistently elevated by more than threefold in bystander cells as opposed to irradiated cells. Conversely, the inhibition of cyclo-oxygenase 2 in this system abrogated the bystander effects. Although the physiologic purpose of the bystander effect remains unclear, several investigators have demonstrated that it results in the proliferation of bystander cells (75), which may represent an evolutionary mechanism of radio-protection whereby lethally damaged cells are replaced by unirradiated (i.e., “undamaged”) cells that proliferate in response to nearby damage.

There is also clear evidence that radiation can affect cells by activating the stromal components and by propagating signaling between the ECM and tumor cells. For example, in vivo animal studies have demonstrated global alterations in ECM components and signaling after radiation (see [47] for review), and numerous studies have demonstrated that decreased sensitivity to both chemotherapeutics and radiation can be correlated to ECM adhesion (see [76] for review). Tissue microarray analyses of biopsies from idiopathic human renal cell carcinomas and renal cell carcinomas arising in patients living more than 19 years after the Chernobyl accident in radiation-contaminated areas of the Ukraine showed a statistically significant decrease or loss and abnormal distribution of major components of the ECM, including fibronectin, laminin, and E-cadherin/ β -catenin, with accompanying increased levels of $p53$ and TGF- $\beta 1$ (77). Direct evidence that irradiated stroma contributes to carcinogenesis was demonstrated through an in vivo study of hemibody irradiation (left vs. right) of mammary fat pads that had been cleared of mammary epithelial cells. Subsequent transplantation of the

weakly tumorigenic balb/c-derived cell line Comma-D cells into the irradiated and unirradiated fat pads led to tumor formation only in irradiated fat pads (78). In general, the published literature supports a model in which tumor formation is suppressed by normal stroma and normal epithelial-mesenchymal interactions and conversely propagated by aberrant epithelial-mesenchymal interactions (see [79–81] for review). Well-studied candidate mediators of radiation-induced aberrant ECM-tumor cell signaling include TGF- β , which is discussed in the previous paragraphs, as well as integrins, a large family of cell adhesion molecules with multiple α and β subunits capable of forming over 20 different transmembrane heterodimers (82,83).

In studies of murine GD25 fibroblasts, prosurvival signaling cascades controlled by the wild-type $\beta 1A$ integrin variant bound to ECM resulted in radiation survival rates in the presence of growth factors that were similar to cell survival rates in growth factor-depleted cultures (84). DNA damage recognition and repair protein complexes are also influenced by integrin-ECM interactions (85,86), suggesting that an integrin-ECM interaction promotes cell survival through increased DNA repair. However, Hodgkinson et al. (87) demonstrated that the ECM- $\beta 1$ -dependent activation of PI3-kinase (PI3K) can override radiation- and etoposide-induced G_2/M cell cycle arrest in small-cell lung cancer cells, thereby allowing the survival of cells with persistent DNA damage, rather than through increased repair or proliferation. Integrins have been shown to be up-regulated selectively after radiation in specific cell types, including endothelial cells, and this relationship has been used to target drugs to tumor vasculature after irradiation in mouse models (88). In addition, the inhibition of $\alpha_5\beta_3$ integrin has been shown to be directly radiosensitizing in human endothelial and lung cancer cell lines (89) and in human glioma, epidermoid, and prostate cancer xenograft models (90).

Translational Radiobiology

Intrinsic Radiation Factors

The experimental work that has perhaps had the single greatest impact on the clinical practice of radiation oncology has come from the field of biomathematics. In particular, mathematical modeling of the dose-response survival curves for each normal tissue has shown that the curves for tissues that manifest early radiation side effects (during treatment or in the weeks following treatment) have similar shapes and slope, as do the curves for tissues that exhibit late radiation side effects (months to years following treatment), but each is distinct. Further, the shapes of the curves for tumors are similar to those for early responding tissues. This interesting discovery led to the hypothesis that late-affected tissues could be spared toxicity by using smaller doses per fraction, thus allowing for dose escalation to the tumor without increasing late effects. This hypothesis has now been validated in numerous phase 3 randomized trials, most notably in patients with head and neck tumors (91), and now governs both the design of future trials of radiation therapy and the daily choices regarding dose and fraction size used in the clinic.

Intrinsic properties of different types of radiation as well as advances in radiation delivery have been used to advantage in the clinic as well. For example, intensity-modulated radiation therapy (IMRT) has dramatically improved the targeting of photon therapy, the particle used most frequently in external-beam radiation. Using this technique, the physician contours on a computed tomography (CT) scan obtained with the patient in the treatment position all structures the dose should be directed to and excluded from and specifies the dose constraints to each volume. A medical dosimetrist then uses computer modeling to optimize a plan that directs multiple mobile blocks into the beam during treatment to create a dose distribution that tightly conforms to the irregular shapes of the target and avoidance structures, which has the effect of minimizing the dose and volume of irradiated normal tissue adjacent to the target. Although this technology is still relatively new, improved tumor control and reduced radiation complications as the result of an improved therapeutic ratio have already been documented in clinical use (see [92] review).

Several centers around the country have expanded their external-beam radiotherapy capability to take advantage of the unique properties of protons. Protons have an advantage over photons in that all of the energy of a proton is deposited at the end of its path. This means one can place the end of this path within the tumor and deliver the maximal desired dose to the tumor without the dose exiting into normal tissues beyond the tumor. This has the obvious benefit of reducing side effects as well as the risk of second malignancies due to low-dose radiation (see [93] for review). Although requiring further study, the radiobiologic effect of photons and protons on mammalian tissue has been shown to be similar (see [93] for review). This permits the body of literature regarding the dose and normal tissue effects of photon therapy to be applied to proton therapy as well.

Sensitizing Tumors to Radiation

Chemotherapy-Enhanced Radiation Therapy

Regarding trials of the combination of chemotherapy and radiation therapy for head and neck cancer, Bernier and Bentzen (94) have made the overarching observation that

the consistent, frequently cited, message from the meta-analyses is that patients with locally advanced tumors achieve a modest, but highly statistically significant survival benefit from concurrent chemo-radiation. . . . whereas neoadjuvant chemotherapy . . . and adjuvant chemotherapy . . . did not [confer] a significant survival advantage.

Indeed, multiple trials in patients with disease across sites have demonstrated that concurrent chemoradiation therapy can improve local control and therefore disease-free survival, although distant metastatic disease is not generally affected. This finding prompted the proposal that combined chemotherapy and radiation therapy should be regarded instead as chemotherapy-enhanced radiation therapy, or CERT, to focus thinking more on how these agents work together (95).

Chemotherapy agents used concurrently with radiation that have been shown to improve outcomes in phase 3 clinical trials

include 5-fluorouracil (now available as an oral prodrug, capecitabine), platinum analogs, gemcitabine, and topoisomerase II inhibitors. Platinum analogs, including cisplatin, carboplatin, and oxaliplatin, are thought to potentiate radiation sensitivity through the formation of platinum-DNA adducts in the presence of radiation-induced free radicals, the inhibition of DNA repair, the increased uptake of platinum, and cell cycle arrest (see [96] for review). Randomized trials in patients with cancer of the cervix (97), head and neck (see [98] for review), esophagus (99), stomach (100), and lung (see [98] for review) have all shown improved clinical endpoints through the addition of concurrent platinum therapy.

The mechanisms of these agents differ, which broadens their "reach" in the treatment of cancer. Gemcitabine is a cytosine arabinoside analog that has potent radiosensitizing activity when given prior to the start of radiation therapy. It makes tumor cells more sensitive to radiation by disproportionately redistributing the cells in the S phase (see [96] for review). Positive results from its use have been seen in trials conducted in patients with primary pancreatic cancer (101), and good response rates when it has been given concurrently with radiation in patients with head and neck cancer have also been reported (102). Unfortunately, the significantly increased radiosensitivity of normal tissues has also been seen in these trials. Topoisomerase II inhibitors such as etoposide also potentiate radiation cell kill when given before or at the start of radiation therapy. These drugs work by inhibiting the enzyme that mediates the cleavage and rejoining of DNA strands to regulate DNA coiling. Although the exact mechanism of the radiation sensitivity induced by topoisomerase II inhibitors has not been determined, Chen et al. (103) have demonstrated that the drug-trapped, cleavable complex generated by these drugs may initiate radiation sensitivity by "interacting" with the replication fork during active DNA synthesis. Such interaction may lead to one of at least three major biochemical events, including double-strand DNA breaks, arrest of the replication fork, and an aborted "cleaved" TOP1-DNA complex (104,105).

The new millennium has marked the beginning of a new era in radiosensitizers-molecular targeted therapies. In general, these inhibitors specifically target the signaling pathways involved in tumor cell growth and progression and are currently being used to augment existing cytotoxic therapy. Many are now being aggressively studied in preclinical and clinical trials and expectations are raised that they will represent new breakthroughs in cancer care (106). The acronym BECT, biologically enhanced cytotoxic therapy, has been coined to refer to these multiagent regimens (107). Such targeted signaling pathway inhibitors now include tyrosine kinase inhibitors such as epidermal (EGFR), vascular endothelial (VEGFR), platelet-derived growth factor (PDGFR) receptor inhibitors; cyclooxygenase-2 inhibitors; mTOR inhibitors; and histone deacetylase (HDAC) inhibitors. What has made these agents attractive as radiosensitizers is that these pathways intersect with pathways involved in the radiation response, such as those that affect cell cycle checkpoints and DNA repair.

Growth Factors

The most mature work investigating the use of targeted molecular therapy in combination with radiation therapy has been in the

area of EGFR inhibitors. EGFR1 is a 170-kD transmembrane glycoprotein belonging to the ErbB family of tyrosine kinases. This family consists of four distinct receptors, with three EGFR inhibitors now approved by the U.S. Food and Drug Administration (FDA) for clinical use: the monoclonal antibody cetuximab (Erbix) and the tyrosine kinase inhibitors gefitinib (Iressa) and erlotinib (Tarceva). Preclinical studies performed in the 1990s in patients with head and neck cancer were the first to demonstrate that EGFR inhibitors sensitize cells to radiation and that radiosensitivity in tumor models is inversely related to EGFR expression. Subsequent *in vivo* studies demonstrated tumor growth delay with these inhibitors (see [98] for review). These data led to clinical phase 1 trials of cetuximab with radiation therapy for patients with head and neck cancer that showed good response rates and acceptable toxicity, the most notable side effect being a maculopapular, acneiform rash in patients who respond (108). A phase III trial in which patients with head and neck cancer were randomized to radiation therapy either with or without cetuximab subsequently showed improved median survival in the cetuximab arm (28–54 months, $p = 0.02$; 109). Additional studies being done through the Radiation Therapy Oncology Group (RTOG) are still accruing patients with head and neck cancer and include RTOG 0234, which is examining the addition of cetuximab to postoperative radiation therapy, and RTOG 0522, which is examining the addition of cetuximab to concomitant boost radiation and cisplatin chemotherapy.

There are strong preclinical data indicating that EGFR inhibition is synergistic or additive in the presence of radiation in other tumors besides head and neck tumors. This is not surprising, since EGFR expression has been documented in most solid tumors, including lung, breast, and colon cancer, in which it is overexpressed in 40% to 60% of tumors (110). EGFR expression has also been associated with human papilloma virus infection in patients with cervical and vulvar cancer, where it is thought to be most relevant in early-stage disease (see [110] for review). In one preclinical study, Bianco et al. (98) demonstrated synergy between ionizing radiation and ZD1839, a small-molecule EGFR inhibitor, in ovarian (OVCAR-3), breast (MCF-7 *adr*), non-small cell lung (A549 and Calu-6), and colon cancer (GEO) cell lines (see [98] for review). In addition, this combination improved survival time in a colon cancer xenograft model. A potential mechanism that accounts for the radiation and chemotherapy sensitization produced by EGFR inhibitors was discovered by Freidmann et al. (see [98] for review), who demonstrated an association between EGFR and DNA-PK in response to treatment with gefitinib in MCF-7 human breast cancer cells. Interestingly, preclinical studies have shown the pharmacokinetics and dosimetry of an auger electron-emitting radiotherapeutic agent targeting EGFR-expressing breast cancer cells now in phase 1 trials (111). Enrollment in a phase 2 study of cetuximab in conjunction with radiation therapy for stage IIIa lung cancer (RTOG 0324) has been closed, but the results have not yet been reported.

EGFR2 (Her2/neu, ErbB2) is a distinct EGFR expressed in multiple tumor types, most notably breast cancer. Her2/neu is amplified in about 25% of breast cancers, and this is associated with worse overall survival. Recent phase III data have

shown that its effect can be overcome through the adjuvant use of the FDA-approved inhibitor trastuzumab (Herceptin; 112). Preclinical data regarding this approach suggest that Her2/neu inhibition may also lead to radiosensitization. Indeed, small-molecule inhibition of Her2/neu signaling had a radiosensitizing effect in human breast cancer cell lines (see [98] for review), which prompted a small clinical trial of concurrent trastuzumab and accelerated hypofractionated radiation therapy in patients with chemoresistant and high-risk breast cancer. Five of seven patients with gross disease in this trial achieved a complete response, and no complete responder or high-risk patient ($n = 15$) showed relapse in the short, 5- to 26-month, follow-up period (113). These intriguing early results suggest that multiple members of the EGFR family can serve as targets for radiosensitization.

Significant inroads have been made in our understanding of the radiosensitization effected by VEGF signaling inhibition through the identification of inhibitors of this pathway (114) and from preclinical data suggesting that the sensitivity of tumor vasculature to radiation is a critical determinant of tumor cell death (20). This latter finding has further heightened interest in these inhibitors as radiosensitizers because of the importance of tumor vasculature to tumor cell survival (115). It has been suggested that even small perturbations in tumor vasculature can translate into significant tumor cell death (116) and that the radioresistance of the tumor vasculature is positively correlated with the radioresistance of the tumor (117), further highlighting the potential importance of these agents. In contrast to the data suggesting that radiation causes endothelial cell death by triggering apoptosis (19,20), Moeller et al. (118) have examined the role of hypoxia inducible factor-1 (HIF-1) in regulating tumor response to ionizing radiation and have found that endothelial cells may undergo significant non-apoptotic death in response to radiation. These authors observed that high HIF-1 activity regulates the secretion of radioprotective cytokines from endothelial cells following radiation therapy. The importance of this regulation was highlighted by the observation of increased tumor control in response to the combination of radiation with a HIF-1 inhibitor (threefold difference in tumor volume 5 days after injection of inhibitor compared with controls, $p < 0.05$; 118).

Conclusion

Our understanding of the molecular regulation of radiation effects has expanded exponentially. Not only have biochemical and molecular findings given us further insight into important elements of classic cellular radiation biology, they have been instrumental in launching an entirely new approach to the radiation treatment of cancer. An increased understanding of the mechanism of radiation resistance has made possible targeted cancer therapy. Clinical investigations of one such agent, cetuximab, have shown improved survival resulting from increasing control of advanced cancer of the head and neck. This proof of principle that the combination of radiation therapy and molecular targeting agents can improve outcome without major toxicity has opened up enormous opportunities for

the treatment of cancer patients, since so many of these agents have shown promise in preclinical studies. Investigations of new targets have hinted at the possibility that we can sensitize tumors and protect normal tissues with a single targeted therapy, thereby

allowing cytotoxic doses of radiation to be delivered even to tumors with classically radioresistant features. Improved physical targeting with x-rays and protons in concert with this novel approach to sensitization holds even greater promise for cancer therapy.

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Hematopoietic Growth Factors and Cytokines

The term “cytokine” refers to a chemical messenger protein that carries a biochemical signal between cells, usually of the immune system, and the rest of the body. *Interleukin* designates any soluble protein or glycoprotein product of leukocytes that regulates the responses of other leukocytes. There are now well over 150 such proteins identified, and it is clear that the pleiotropic nature of many cytokines and interleukins allows them to influence virtually all organ systems. In addition to their vital role in promoting hematopoietic cell growth, differentiation, and activity, these molecules are vital to the proper functioning of the central nervous system, cardiorespiratory system, and liver, as well as to bone remodeling, lipid metabolism, and embryogenesis and maintenance of pregnancy. Interestingly, many of these molecules have pleiotropic effects on numerous organ systems. For example, stem cell factor influences hematopoiesis and neurogenesis, and prolactin promotes multiproduction and erythropoiesis. Cytokines may have their own private receptor but may also share a “public” receptor with other cytokines (Table 51-1; 1,2), perhaps explaining some of the redundancy in their effects (Figure 51-1).

The identification and cloning of hematopoietic growth factors and cytokines have revolutionized medical practice. Raising white blood cell counts in patients with neutropenia was unimaginable until the discovery of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Today, growth factors are routinely used to alleviate neutropenia and, to a lesser extent, thrombocytopenia and anemia after chemotherapy. They can also help mobilize stem cells for transplantation and they may have the potential to mobilize the immune system against infection or cancer.

Herein, we give an overview of the biologic characterization of the known clinically relevant interleukins and selected cytokines, the rationale for their use in therapy for patients with cancer, and the clinical experience with them.

Erythropoietin

Erythropoietin (EPO) is the most important hormone regulator of erythropoiesis. It has an accepted place in the treatment of anemia caused by a variety of illnesses (Table 51-2; 3). Because its primary production source is the kidney, it is not surprising that EPO has its best established role in the treatment of anemia

due to the EPO-deficient state in kidney disease. However, many patients with anemia due to cancer also have a relative deficiency in endogenous EPO and respond to EPO. Interestingly, certain cases of familial erythrocytosis have been attributed to the presence of EPO-hypersensitive cells. This heightened EPO response results from the formation of a truncated EPO receptor that is missing a negative regulatory domain.¹

EPO: Clinical Trials/Applications

EPO is most useful in those anemias in which there is an absolute or a relative deficiency in endogenous EPO levels, such as in renal failure and cancer, respectively (Table 51-2). The quality of life for patients with anemia due to renal failure or cancer who respond to EPO is clearly improved. Although EPO has generally been used for patients with a hemoglobin below 10 gm/dL, the maximum improvement in the quality of life actually occurs in patients with higher hemoglobin levels (11–13 gm/dL; 1). In the case of cancer, however, not all patients respond, and those with the highest levels of endogenous EPO are probably less likely to benefit. A recently discovered complicating factor to defining optimal EPO treatment has been the finding of decreased survival in some patients treated in randomized trials with “optimization” of hemoglobin (4).

Granulocyte-Macrophage Colony-Stimulating Factor

Autonomous production by the tumor of GM-CSF (or G-CSF) has also been implicated as a pathophysiologic mechanism underlying leukemoid reactions in cancer patients. GM-CSF is used clinically for the treatment of neutropenia after chemotherapy or transplantation, for treatment of graft failure, and for peripheral blood stem cell mobilization (Table 51-2; 5,6).

GM-CSF: Clinical Trials/Applications

GM-CSF is safe and effective in the treatment of patients with acute myelogenous leukemia (AML) who are undergoing induction therapy. GM-CSF decreases the neutropenic period and the rate of serious infections in the elderly. This molecule is also indicated for accelerating myeloid reconstitution after allogeneic

Table 51-1 Receptors, Natural Antagonists, and Chromosomal Locations of Growth Factors and Cytokines

	Receptor	Natural Antagonists	Chromosomal Locations
Erythropoietin (EPO)	EPO receptor	Soluble EPO receptor	7q21
GM-CSF	Type I receptor with α and β subunits		5q31.1
G-CSF	G-CSF receptor		17q11.2-q12
M-CSF	Fms		1p21-p13
Stem cell factor	c-kit	Soluble c-kit receptor	12q22-12q24
Thrombopoietin	Mpl		3q27-q28
IL-1	IL-1RI and IL-1RII?Extended family of 10 members including IL-18R)	Soluble IL-1RI and IL-1RII and IL-1RA	2q13
IL-2	$\alpha\beta\gamma$ heterotrimeric complex		4q26-q27
IL-3	IL-3 receptor (heterodimer of IL-3 specific α subunit and β subunit)		5q31
IL-4 and IL-13	IL-4 and IL-13 receptors share subunits Type I IL-4 receptor (IL-4R α and IL-2 receptor γ chain subunits) transduces IL-4; type II IL-4 receptor (IL-4R α and the IL-13R α 1 subunits) transduces IL-4 and IL-13; IL-4R α and IL-13R α 2 complex or two IL-13R α transduce IL-13	Soluble IL-4 and IL-13 receptors exist	5q31
IL-5	Consists of IL-5R α (IL-5-specific) and a β subunit. β subunit is common to IL-3 and GM-CSF complexes		5q31
IL-6	IL-6R α together with gp130		7p21
IL-7	Composed of IL-7R α (CD127) and the common γ chain subunits		8q12-q13
IL-8	IL-8R α and IL-8R β exist		4q12-q13
IL-9	IL-9 receptor		5q31.1
IL-10	IL-10 receptor interferon receptors		1q31-q32
IL-11	IL-11R α and gp130 subunits gp130 = CD130 on 5q11 IL-6, oncostatin M, and leukemia inhibitory factor also use gp130 subunit		19q13.3-q13.4
IL-12	IL-12R β 1 and IL-12R β 2 chains are related to gp130	IL-12 p40 homodimers	IL-12A: 3p12-q13.2 IL-12B: 5q31.1-q33.1
IL-15	High affinity receptor requires IL-2R β and γ chains and IL-15R α chain		4q31
IL-16	Requires CD4 for biologic activities Tetraspanin CD9		15q26.1
IL-17	IL-17 receptor		2q31
IL-18	IL-18 receptor	IL-18 binding protein exists	11q22.2-q22.3
IL-19	IL-20R1 and IL-20R2		1q32
IL-20	IL-20R1 and IL-20R2		1q32
IL-21	IL-21 receptor		4q26-27
IL-22	IL-22R1 and IL-10R2		12q14
IL-23	IL-12Rb1 and IL-23R		12q13
IL-24	IL-20R1 and IL-20R2 IL-22R1 and IL-20R2		1q32
IL-25	IL-17BR		14q11

Table 51-1 Receptors, Natural Antagonists, and Chromosomal Locations of Growth Factors and Cytokines—Continued

	Receptor	Natural Antagonists	Chromosomal Locations
IL-26	IL-20R1 and IL-10R2		12q14
IL-27	TCCR/WSX-1 and GP130		12q13
IL-28A, 28B, and 29	IL-28R1 and IL-10R2		19q13
IL-31	IL-31receptor A and oncostatin M receptor		12q24
IL-32	Proteinase 3		16p13.3
IL-33	ST2		9p24.1

IL, interleukin.

bone marrow transplantation. It also increases survival in patients who have engraftment failure or delay after allogeneic or autologous transplantation, and it can be exploited to enhance stem cell mobilization for transplant.

Granulocyte Colony-Stimulating Factor

G-CSF has revolutionized the treatment of neutropenia and its sequelae (infection). It has been used by millions of patients worldwide and is remarkably effective and virtually devoid of side effects (Table 51-2; 7). Some patients with solid tumors present with significantly increased leukocyte counts due to G-CSF secretion. Finally, point mutations in the gene for the G-CSF receptor have been described anecdotally in patients with AML that evolved from severe congenital neutropenia.

G-CSF: Clinical Trials/Applications

G-CSF promotes a rapid increase in neutrophilic leukocytes, which lasts about 24 hours. Despite the multitude of patients who

have received G-CSF, few side effects have been reported. Even very long-term G-CSF administration seems fairly innocuous; the most common toxicity is bone pain.

A summary of current American Society for Clinical Oncology (ASCO) guidelines suggests that CSF can be used for primary prophylaxis of febrile neutropenia after chemotherapy if the risk is about 20%. It is also recommended for patients at high risk, based on age, medical condition, disease characteristics, and myelotoxicity of chemotherapy. Prophylaxis is also recommended for diffuse aggressive lymphoma in patients older than 65 treated with curative therapy. Patients exposed to lethal radiotherapy should also receive CSF (8,9). In the transplantation setting, the administration of G-CSF reduces neutropenia and infection. G-CSF also mobilizes autologous peripheral blood progenitor cells; these cells are used to accelerate hematopoietic recovery in patients who have received myeloablative or myelosuppressive chemotherapy. Finally, in patients with acquired immune deficiency syndrome (AIDS), G-CSF reverses and prevents zidovudine-induced neutropenia (7). Of interest, G-CSF may also be useful in enhancing the defenses of non-neutropenic patients with AIDS who have bacterial infections.

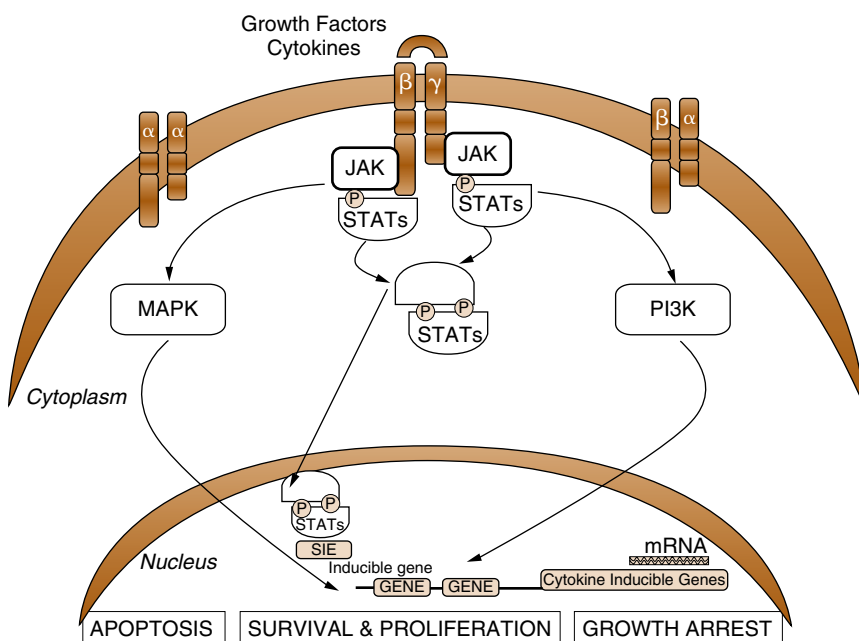


FIGURE 51-1 Actions of cytokine/growth factors binding to their receptors on the cell surface. Cytokines and growth factors are the messengers that mediate intercellular communication. The regulation of cellular and nuclear functions by cytokines and growth factors is initiated through the activation of cell surface receptors. All receptors have a ligand-binding domain that ensures ligand specificity and an effector domain that initiates the generation of the biological response upon ligand binding. The activated receptor may then interact with other cellular components to complete the signal transduction process. Briefly, cytokine binding to receptor subunits induces homo- or heterodimerization resulting in the activation of Jaks that are bound to the receptor chains. The Jaks in turn phosphorylate tyrosine-based docking sites on the receptor. STATs bind via their SH2 domains. The STATs are then phosphorylated, form homo- or heterodimers, translocate to the nucleus, where they bind target sequences, thereby regulating gene expression.

Table 51-2 Growth Factors and Cytokines in the Clinic^a

Major Clinical Trials	
Erythropoietin (EPO)	• Anemia of renal failure • Anemia of zidovudine therapy of HIV (with endogenous EPO level < 500 mU/mL) • Anemia of cancer, especially after chemotherapy of solid tumors • Reduction of blood transfusions in elective surgery • Potentiation of autologous blood donation • Anemia of prematurity • Maximum quality-of-life improvement is at hemoglobin of 11-13 gm/dL. • Hyperglycosylated EPO (darbopoietin alfa) has prolonged half-life and can be administered less frequently. • Postulated to offer neuroprotection after neurologic damage since EPO/EOP receptors are present in the central nervous system
GM-CSF	• Neutropenia due to myelosuppressive chemotherapy or bone marrow transplantation (BMT) • Peripheral blood stem cell mobilization • Graft failure • After induction therapy for acute myelocytic leukemia (AML)
G-CSF	• Neutropenia due to chemotherapy or BMT • Chronic and cyclic neutropenia • AIDS-related neutropenia • Autoimmune neutropenia • Peripheral blood stem cell mobilization • G-CSF reduces morbidity from high-risk febrile neutropenia treated with antibiotics
M-CSF	• Enhances hematopoietic recovery after chemotherapy or transplantation • Attenuates neutropenia in chronic neutropenia • Lowers serum cholesterol • May be useful in therapy of fungal infections
Stem cell factor (SCF)	• Peripheral blood progenitor mobilization (SCF + G-CSF better than SCF alone) • Aplastic anemia (trilineage responses seen after SCF)
Thrombopoietin	• Accelerates platelet recovery after chemotherapy • Increases platelet yield from normal donors for platelet transfusions • Enhances mobilization of peripheral blood progenitor cells by G-CSF • Nonimpressive effects on platelet recovery after myeloablative therapy
IL-1	IL-1α and IL-1β • Modest reduction in postchemotherapy neutropenia or thrombocytopenia; numerous side effects • No significant antitumor activity in melanoma or renal cell carcinoma IL-1RA • No clear-cut reduction in mortality in sepsis patients • Amelioration of rheumatoid arthritis and graft-versus-host disease
IL-2	• Antitumor activity in melanoma and renal cell carcinoma • IL-2 diphtheria fusion toxin (DAB-IL-2) approved for used in cutaneous T-cell lymphomas
IL-3	• Increases stem cell mobilization when used with G-CSF or GM-CSF • In combination with GM-CSF, hastens bone marrow recovery after transplant • Sequential IL-3 and GM-CSF produces multilineage responses in some marrow failure patients • Induces occasional sustained remissions in Diamond-Blackfan anemia
IL-4 and IL-13	• Only minor antitumor activity has been seen a variety of human cancers of IL-4
IL-5	• IL-5 antagonists may be useful in treatment of allergy and asthma. However, trial of monoclonal antibody against IL-5 was not effective in asthma
IL-6	• Response rates 8%–14% in melanoma and renal cell carcinoma • Modest platelet-enhancing ability after chemotherapy or autologous transplant with significant toxicity • Antibody to block IL-6 is entering clinical trial
IL-8	• Antibodies to block IL-8 are entering clinical trial
IL-10	• Trend toward efficacy in rheumatoid arthritis, inflammatory bowel disease, and autoimmune diseases
IL-11	• Approved for use to prevent chemotherapy-induced thrombocytopenia
IL-12	• Potential use in vaccine development • No benefit in hepatitis C trial • Modest antitumor activity with significant toxicity in melanoma and renal cell carcinoma
IL-16	• May have potential use in HIV infection
IL-20	• May have potential for chronic inflammatory skin disease

Table 51-2 Growth Factors and Cytokines in the Clinic^a—Continued

Major Clinical Trials	
IL-21	• Since it controls adaptive immune responses, its use in a clinical setting may prove efficacious for the treatment of cancer and infectious disease
IL-24	• Clinical trials for the treatment of melanoma are underway
IL-25	• Potent inflammatory activity and its association with various human disease states suggest this cytokine family as an important contributor to the pathophysiology of pulmonary diseases
IL-28A, 28B, and 29	• Alternative therapeutic choice to type I IFNs
IL-32	• Potential therapeutic target in rheumatoid arthritis

AIDS, acquired immunodeficiency syndrome; EOP G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; HIV, human immunodeficiency virus; IL, interleukin.

^a Many of the listed applications refer to clinical trials and, are not approved uses.

However, studies have shown only modest benefit for G-CSF in the setting of nonneutropenic infection in normal individuals.

Macrophage Colony-Stimulating Factor

M-CSF affects a variety of organ systems, but its cardinal effect remains its ability to influence most aspects of monocyte/macrophage development and function (Table 51-3; 10). In addition to its hematopoietic effects, M-CSF and Fms (the M-CSF receptor) are expressed in the brain. This cytokine induces microglial proliferation, activation, and survival. In malignancy, mutations in Fms have been reported at codon 969 in about 10% of cases of human myeloid malignancies.

M-CSF: Clinical Trials/Applications

M-CSF given to patients with AML after consolidation chemotherapies shortened the periods of neutropenia and thrombocytopenia after chemotherapy and reduced the incidence and shortened the duration of febrile neutropenia (10). Similar salutary effects have been reported after chemotherapy or bone marrow transplantation. M-CSF can elevate neutrophil counts in children with chronic neutropenia.

Stem Cell Factor

Stem cell factor (SCF) is also known as kit ligand, mast cell growth factor, or steel factor. It functions as a hematopoietic cytokine that triggers its biologic effect by binding to c-kit (the SCF receptor; Table 51-3; 11–14). The average concentration of SCF in normal human serum is 3.3 ng/mL. Serum SCF concentrations are not elevated in patients with aplastic anemia, myelodysplasia, or chronic anemia or after marrow ablative therapy. Thus, the level of SCF in the circulation, unlike the level of EPO, is not inversely related to the number of hematopoietic cells. Alterations in the local distribution of SCF within the skin have been implicated in the pathogenesis of cutaneous mastocytosis (1). Point mutations in the c-kit receptor cytoplasmic domain have been identified in murine and human mast cell lines and in hematopoietic cells from patients with mast cell disorders. Finally, activating mutations

in kit, a kinase receptor, characterize a type of leiomyosarcoma known as gastrointestinal stromal tumors. This finding has led to new targeted therapies of tremendous impact.

SCF: Clinical Trials/Applications

SCF factor seems to be reasonably well tolerated by patients, with the predominant side effects being transient local erythema and long-lasting hyperpigmentation at injection sites. The most worrisome toxicity is a mast cell effect resulting in allergic-like reactions characterized by urticaria, with or without respiratory symptoms (1).

Of special interest is the role of mutations in the SCF receptor (kit) in gastrointestinal stromal tumors. These mutations activate the kinase enzymatic activity of kit. Kinase inhibitors targeted against kit (imatinib and sunitinib) have been found to be dramatically effective in these notoriously chemotherapy-resistant tumors (12).

Thrombopoietin

The cytokine basis of megakaryocyte and platelet production has been more enigmatic than that of other lineages (Table 51-3; 15–22). Factors that have now been implicated in at least some aspects of thrombocyte development include interleukin-3 (IL-3), IL-6, IL-9, IL-11, G-CSF, GM-CSF, SCF, leukemia inhibiting factor, and thrombopoietin (TPO). The latter molecule is believed to be of paramount importance in the physiologic regulation of platelet production. Unfortunately, however, compared with the striking effects of the granulopoietic factors in neutropenic patients, use of the thrombopoietic molecules in the clinic setting has been disappointing. It has been suggested that the temporal pace of thrombopoietic response is physiologically ordained to be considerably slower than myelopoietic response, and that may explain why short courses of thrombopoietins seem to be ineffective (9).

TPO: Clinical Trials/Applications

Two forms of TPO have entered clinical trials (16): (1) TPO (the full-length polypeptide) and (2) polyethylene glycol (PEG)-conjugated recombinant human megakaryocyte growth and

Table 51-3 Major Biologic Activities of Growth Factors and Cytokines

	Biologic Activities
Erythropoietin (EPO)	• Promotes the proliferation, differentiation, and survival of erythroid precursors
GM-CSF	• Stimulates growth of multilineage progenitors, BFU-E, granulocyte, macrophage, and eosinophil colonies • Induces migration and proliferation of vascular endothelial cells • Activates mature phagocytes (neutrophils, macrophages, eosinophils)
G-CSF	• Regulates production and function of neutrophils
M-CSF	• Influences most aspects of monocyte/macrophage development and function • Stimulates hematopoiesis • Induces osteoclast production • Helps maintain pregnancies • Lowers cholesterol levels • Affects microglial function
Stem cell factor	• Promotes hematopoiesis at multiple levels • Influences primordial germ cell and melanocyte migration during embryonic life • Affects immunoregulatory cells (B and T cells, mast cells, natural killer [NK] cells, dendritic cells) • Influences hematopoietic cell adhesive properties
Thrombopoietin	• Major regulator of platelet production • Acts in synergy with EPO to stimulate growth of erythroid progenitors • Acts in synergy with IL-3 and SCF to stimulate proliferation and prolong survival of hematopoietic stem cells
IL-1	• Induces production of multiple cytokines • Up-regulates cell-surface cytokine expression • Synergizes with other cytokines to stimulate hematopoietic progenitor proliferation • Influences immune regulation (T- and B-cell responses) • Modulates endocrine function • Affects bone formation • IL-1R acts as a cofactor in neural transmission • IL-1 is probably not critical for normal hematopoiesis; it is, however, central in disease states
IL-2	• Induces proliferation and activation of T cells, B cells, and NK cells
IL-3	• Stimulation of multilineage hematopoietic progenitors, especially when used in combination with other cytokines (SCF, IL-1, IL-6, G-CSF, GM-CSF, EPO, TPO)
IL-4 and IL-13	• Both IL-4 and IL-13 are involved in allergic reaction (induce switch to IgE)
IL-5	• Regulates production, function, survival, and migration of eosinophils • Enhances basophil number and function
IL-6	• B- and T-cell development and function • Thrombopoiesis • Acute-phase protein synthesis • Inhibition of hepatic albumin excretion • Osteoclastic bone resorption • Neural differentiation
IL-7	• Critical for T- and B-cell development
IL-8	• Potent chemoattractant agent for a variety of leukocytes, especially neutrophils • Suppresses colony formation of immature myeloid progenitors • Increases keratinocyte and endothelial cell proliferation • Increases adhesiveness of melanoma cells
IL-9	• Supports clonogenic maturation of erythroid progenitors • Acts as a mast cell differentiation factor • Protects lymphomas from apoptosis • Cooperates with IL-4 in B-cell responses • Enhances neuronal differentiation
IL-10	• Inhibits cytokine synthesis by Th1 cells and monocytes/macrophages • Stimulates B cell proliferation • Involved in transformation of B cells by Epstein-Barr virus and tumor necrosis factor (TNF) receptors
IL-11	• Best known as a thrombopoietic factor • Stimulates multilineage progenitors, erythropoiesis, myelopoiesis, and lymphopoiesis • Decreases mucositis in animal models • Stimulates osteoclast development • Inhibits adipogenesis • Stimulates proliferation of neuronal cells

Table 51-3 Major Biologic Activities of Growth Factors and Cytokines—Continued

	Biologic Activities
IL-12	• Proinflammatory cytokine important in resistance to infections • Th1 development • Stimulatory and inhibitory effects on hematopoiesis
IL-15	• Triggers proliferation and immunoglobulin production in preactivated B cells • Number of CD8⁺ memory T cells may be controlled by balance of IL-15 (stimulatory) and IL-12 (inhibitory) • Stimulates proliferation of NK cells and activated CD4⁺ or CD8⁺ T cells • Facilitates the induction of LAK cells and CTLs • Stimulates mast cell proliferation • Promotes proliferation of hairy-cell leukemia and chronic lymphocytic leukemia cells
IL-16	• Chemoattractant for CD4⁺ cells (T cells, monocytes, eosinophils) • May be involved in asthma and in granulomatous inflammation • Has antiviral effects on HIV-1
IL-17	• May mediate, in part, T-cell contribution to inflammation • Stimulates epithelial, endothelial, fibroblastic, and macrophage cells to express a variety of inflammatory cytokines • Promotes the capacity of fibroblasts to sustain hematopoietic progenitor growth • Promotes differentiation of dendritic cell progenitors • May be involved in the pathogenesis of rheumatoid arthritis and graft rejection
IL-18	• Promotes production of IFN-γ, TNF • Targets are T cells, NK cells, and macrophages • Promotes Th1 responses to virus
IL-19	• Induces IL-6 and TNF-α
IL-20	• Induction of genes involved in inflammation such as TNF-α, MRP14 and MCP-1
IL-21	• Mainly, regulates T-cell proliferation and differentiation • Regulates cell-mediated immunity and the clearance of tumors
IL-22	• Up-regulates the production of acute-phase reactants • Induces the production of ROS in resting B cells
IL-23	• A unique function of IL-23 is the preferential induction of proliferation of the memory subset of T cells
IL-24	• Induces IL-6, TNF-α, IL-1b, IL-12 and GM-CSF • Functionally it has opposite effects with IL-10 • Infection with Ad-IL24 results in down-regulation of Bcl-2 and Bcl-XL (anti-apoptotic proteins) and up-regulation of Bax and Bak (pro-apoptotic proteins) in cancer cells
IL-25	• IL-25 induces IL-4, IL-5, and IL-13 gene expression and protein production
IL-26	• Immune-protective role against viral infection
IL-27	• Early Th1 initiation. • Synergizes with IL-12 in inducing IFN-γ production by T cells and NK cells
IL-28A, 28B, and 29	• Antiviral activities
IL-31	• Responsible for promoting the dermatitis and epithelial responses that characterize allergic and nonallergic diseases
IL-32	• Induces other proinflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, and IL-8 • Induces IκB degradation • Phosphorylates p38 MAPK signaling pathway
IL-33	• Activates NF-κB and MAP kinases • Drives production of Th2-associated cytokines from in vitro polarized Th2 cells • Induces the expression of IL-4, IL-5, and IL-13 • Leads to severe pathologic changes in mucosal organs

AIDS, acquired immunodeficiency syndrome; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; HIV, human immunodeficiency virus; IL, interleukin.

development factor (PEG-rHuMGDF). Because its biologic action is prolonged, parenteral administration of TPO for 7 to 10 days increases platelet production 6 to 16 days later (17). Results of clinical trials of PEG-rHuMGDF or recombinant human TPO in patients with cancer who were receiving chemotherapy, albeit with regimens that produce only moderate thrombocytopenia, suggest that platelet counts return to baseline significantly faster and that

the nadir platelet counts are higher (18). However, the effectiveness of these molecules in accelerating platelet recovery after myeloablative therapy has not been impressive (19). Furthermore, in patients with delayed platelet recovery after peripheral-blood stem cell or bone marrow transplantation, recombinant human TPO did not significantly raise platelet counts in most patients (20). TPO can result in multilineage mobilization of peripheral blood

progenitor cells. The kinetics of progenitor release differs from that after G-CSF. Following G-CSF, peripheral blood progenitors increase almost immediately, peak at day 5 to 6, and decrease with G-CSF cessation. In contrast, PEG-MGDF resulted in a late and sustained increase in progenitors, with levels first detected on day 8 and climbing on day 12, despite cytokine discontinuation (22). PEG-rHuMGDF has also been given to healthy subjects in a single dose of 3 mg/kg of body weight. Administration of this molecule increased the yield of platelets by a factor of nearly 4 and was associated with a quadrupling of platelet counts in the recipients of the apheresed platelets (21).

Interleukin-1

Interleukin-1 (IL-1 α and IL-1 β) is the prototypic multifunctional cytokine (Table 51-3; 23–28). This molecule influences nearly every organ system. Because IL-1 is a highly inflammatory cytokine, the margin between salutary effects and serious toxicity is exceedingly narrow.

High levels of IL-1 are seen in patients with infections (viral, bacterial, fungal, and parasitic), intravascular coagulation, and cancer (both solid tumors and hematologic malignancies). IL-1RA, a naturally occurring receptor antagonist, may also be dysregulated in inflammatory and neoplastic disease. Ultimately, it is the balance between agonist and antagonist that is probably important in determining disease manifestation.

IL-1: Clinical Trials/Applications

IL-1 α and IL-1 β have also been administered in clinical trials, mainly involving cancer patients (23). In general, the acute toxicities of either isoform of IL-1 were greater after intravenous injection than after subcutaneous injection. Subcutaneous injection was associated with significant local pain, erythema, and swelling. Dose-related chills and fever were observed in nearly all patients, and even a 1-ng/kg dose was pyrogenic. Nearly all patients receiving intravenous IL-1 at doses of 100 ng/kg or greater experienced significant hypotension, probably because of induction of nitric oxide. IL-1RA has recently been approved by the U.S. Food and Drug Administration (FDA) for the treatment of rheumatoid arthritis (29).

Interleukin-2

IL-2 acts as a T-cell growth and activation factor, but B cells, NK cells, and lymphokine-activated killer cells also respond to this cytokine (Table 51-3; 30). Following binding of IL-2 with the trimeric receptor complex, internalization occurs and cell-cycle progression is induced in association with the expression of a defined series of genes. A second functional response occurs through the IL-2 $\beta\gamma$ dimeric receptor, also known as the intermediate affinity dimeric complex (kD, 10⁻⁹), and involves the differentiation of several subclasses of lymphocytes into lymphokine-activated killer (LAK) cells (31). This response occurs in patients with cancer who receive IL-2 (32,33) and was originally considered to be a critical

part of the anticancer effect of IL-2. LAK cells recognize and kill tumor cells, regardless of the histocompatibility expression status on fresh human tumor cells tested (34). The multiple biologic effects of IL-2 on immune cells include the induced proliferation of antigen-stimulated T cells and induction of cytotoxicity in major histocompatibility complex (MHC)–restricted, antigen-specific T-lymphocytes, NK cells leading to non-MHC–restricted LAK cell activity, and activation of tumoricidal monocytes. It is not clear what roles any of these effector systems have in vivo (35).

IL-2: Clinical Trials/Applications

IL-2 has antitumor activity in diseases such as melanoma and kidney cancer, albeit with relatively low response rates and at the cost of considerable toxicity. For instance, overall response rates of renal cell cancer to IL-2 are in the range of 15% to 25%, with a complete remission rate of 5% to 10%. Complete response rates and response duration seem to favor high-dose rather than low-dose regimens. In melanoma biochemotherapy, regimens combining IL-2 and interferon- α with, for instance, cisplatin, vinblastine, and dacarbazine produce response rates of up to 60%, but this has yet to be translated into a confirmed survival effect (30). IL-2 has also been given to leukemic patients in a variety of doses and schedules, with hints that it might be useful in remission maintenance (30). Development of second-generation IL-2 analogues that do not induce the same high levels of secondary cytokines provides promise for further reduction of the toxicities, providing that the efficacy is not dependent on these secondary effects (36). Another approach to therapy has been to use IL-2 attached to a toxin to target and kill cancer cells bearing the IL-2 receptor. DAB389IL-2 is an IL-2 receptor (IL-2R)–specific fusion protein. It contains the enzymatic and translocation domains of the diphtheria toxin fused to human IL-2. This chimera is able to direct the cytotoxic action of the diphtheria toxin enzymatic region only to cells that bear the IL-2R. DAB389IL-2 has been approved for treatment of cutaneous T-cell lymphomas that are CD25 (IL-2 receptor) positive. Antitumor effects may also be seen in patients with other lymphoid diseases bearing the IL-2 receptor (1).

Interleukin-3

IL-3 stimulates multilineage hematopoietic progenitors (Table 51-3; 37–40). In vitro data from supernatants of long-term bone marrow cultures suggest that marrow stromal cells produce reduced levels of IL-3 in patients with aplastic anemia (1). IL-3 has also been implicated in patients with acute lymphocytic leukemia (ALL) with at (5:14)(q31;q32) translocation (1). In two such patients, the translocation resulted in juxtaposition of the IL-3 gene and the Ig heavy-chain gene, and excess IL-3 transcripts were produced by the leukemic cells, perhaps explaining the eosinophilia seen in these patients.

IL-3: Clinical Trials/Applications

IL-3 has been studied in clinical trials of peripheral blood stem cell mobilization, as well as for post-chemotherapy and

post-transplantation cytopenias and for bone marrow failure states. Most studies have shown only modest effects of IL-3 by itself; in conjunction with other growth factors, however, significant benefits have been demonstrated. For instance, in patients with bone marrow failure treated with IL-3 followed by GM-CSF (IL-3 dosages of $>1.2 \mu\text{g/kg/d}$), seven (44%) of 16 patients with severe pancytopenia had multilineage responses with normalization or near-normalization of blood counts. Although prolonged therapy was necessary to achieve maximal hematopoietic recovery, responses were durable for up to 4 years after discontinuation of treatment (39,41). Side effects of IL-3 include dose-dependent fever, rash, fatigue, diarrhea, rigor, musculoskeletal pain, chills, headache, conjunctivitis, edema, chest pain, dyspnea, decreases in platelet counts, increases in basophilic counts, marrow fibrosis, and pulmonary edema. The tolerance to IL-3 seems to be several-fold better in patients with bone marrow failure states compared with those treated after chemotherapy (39).

Interleukin-4 and Interleukin-13

IL-4 and IL-13 are closely related (42). They share biologic and immunoregulatory functions on B cells, monocytes, dendritic cells, and fibroblasts. The major regulatory sequences in the IL-4 and IL-13 promoters are identical, thus explaining their restricted expression pattern in activated T cells and mast cells. Furthermore, the IL-4 and IL-13 receptors are multimeric and share at least one common chain, IL-4RA. This, together with similarities in IL-4 and IL-13 signal transduction, explains the remarkable overlap of biologic properties between these two cytokines (Table 51-3; 42–44). The inability of IL-13 to regulate T-cell differentiation due to a lack of IL-13 receptors on T lymphocytes, however, represents a major difference between these cytokines. Therefore, despite the impact redundancy of these two molecules, regulatory mechanisms are in place to guarantee their distinct functions.

IL-4: Clinical Trials/Applications

Despite the preclinical promise of IL-4, to date clinical trials of this molecule have found the molecule to be safe and nontoxic in humans but with only sporadic antitumor activity (44).

Interleukin-5

IL-5 is a T-cell–derived cytokine involved in the pathogenesis of atopic diseases. It specifically controls the production, activation, and localization of eosinophils. Eosinophils mediate allergic and asthmatic symptoms. T cells purified from the bronchoalveolar lavage and peripheral blood of persons with asthma secrete an elevated amount of IL-5. Therefore, agents that suppress the production or activity of IL-5 would be expected to ameliorate the pathologic effects of the allergic response (Table 51-3; 45). Interestingly, IL-5 is secreted from Reed-Sternberg cells and may therefore be the cause of eosinophilia in patients with Hodgkin disease (1).

Interleukin-6

IL-6 exhibits functional pleiotropy and redundancy (Table 51-3; 46–51). IL-6 is involved in the immune response, inflammation, and hematopoiesis. Indeed, prior to its complete characterization, this molecule was variously referred to as interferon- β 2, B-cell stimulatory factor 2, human plasmacytoma growth factor, or hepatocyte stimulatory factor. The biologic effects of IL-6 include synthesis of acute phase reactants in the liver, as well as effects on the hypothalamic-pituitary axis, bone resorption, and both the humoral and cellular arms of the immune system (1). As a major inducer of the acute-phase response, this cytokine may play a role in the pathogenesis of sepsis. IL-6 acts as a growth factor for myeloma/plasmacytoma, keratinocytes, mesangial cells, renal cell carcinoma, and Kaposi sarcoma and hematopoietic stem cells. On the other hand, IL-6 also inhibits the growth of myeloid leukemic cell lines and certain carcinoma cell lines.

IL-6 has been implicated as a mediator of B symptoms in lymphoma (47). Elevated serum IL-6 concentrations have also been associated with an adverse prognosis both in Hodgkin lymphoma and in non-Hodgkin lymphoma (NHL; 46,48–50). In diffuse large-cell lymphoma, IL-6 levels were found to be the single most important independent prognostic factor selected in multivariate analysis for predicting complete remission rate and relapse-free survival (50). IL-6 levels may also be exploitable as a prognostic factor in numerous solid and hematopoietic cancers.

IL-6: Clinical Trials/Applications

In patients undergoing chemotherapy or autologous transplantation, IL-6 has minimal to no platelet-enhancing activity at tolerable doses. Toxicity includes fever and anemia (1). IL-6 has also been tested as an antitumor agent in melanoma and renal cell carcinoma. Response rates have been low ($<15\%$). IL-6 inhibitors have entered the clinic. An antibody against IL-6 receptor (MRA) has been approved in Japan for the treatment of Castleman disease. An antibody against IL-6 (CNT0328) is being studied in the United States, and preliminary results show encouraging activity in lymphoma and Castleman disease.

Interleukin-7

IL-7 promotes the proliferation of B-cell progenitors in the absence of stromal cells (Table 51-3; 52–55). It is secreted by stromal cells in the bone marrow and thymus and is irreplaceable in the development of both B and T cells (1). High IL-7 levels are found in states of T-cell depletion and may therefore play a role in promoting T-cell expansion (55). High levels of IL-7 are also found in chronic lymphocytic leukemia and in Burkitt lymphoma, and transgenic mice overexpressing the IL-7 gene show dramatic changes in lymphocyte development, which can result, in some instances, in the formation of lymphoid tumors (1).

Interleukin-8

IL-8 is a potent, proinflammatory chemokine that induces trafficking of neutrophils across the vascular wall (chemotaxis) (Table 51-3; 56,57). This molecule belongs to a chemokine superfamily that includes neutrophil-activating peptide-2, platelet factor-4, growth-related cytokine (GRO) and interferon inducible protein-10, all of which are responsible for the directional migration of various cells (56). Interestingly, IL-8 receptor demonstrates strong homology to a gene encoded by human herpesvirus-8 (HHV-8; implicated in the etiology of Kaposi sarcoma; 1). IL-8 can induce tumor growth, an effect attributed to its angiogenic activity, a property that promotes vascularization. On the other hand, antitumor effects of IL-8 have also been reported. Of interest in this regard is the fact that increased levels of IL-8 have been observed in lung carcinomas and in melanomas. IL-8 may be a growth factor for pancreatic cancer and for melanoma (56). In melanomas, IL-8 levels correlate with the growth and metastatic potential of the tumor cells, and exposure of the cells to interferon decreases IL-8 levels and cancer cell proliferation (57). Blocking IL-8 or IL-8R has been suggested as a therapeutic strategy (56).

Interleukin-9

Human IL-9 was originally identified as a mitogenic factor for a human megakaryoblastic leukemia. More recently, IL-9 targets were found to encompass a wide range of cells (Table 51-3; 58,59). There is an interesting paradox between the unresponsiveness of normal T cells to IL-9 and the potent activity of this molecule on lymphoma cells. This contrast is illustrated by the observation that murine T cells acquire the ability to respond to IL-9 after a long period of in vitro culture, while they simultaneously acquire characteristics of tumor cell lines. Observations made with transgenic mice also demonstrate the oncogenic potential of dysregulated IL-9 production, since 5% to 10% of mice that overexpress this cytokine develop lymphoblastic lymphomas (59). In line with these findings, constitutive IL-9 production by human Hodgkin lymphomas and large-cell anaplastic lymphomas has now been clearly documented (58).

Interleukin-10

IL-10 is a pleiotropic cytokine, initially discovered as an activity produced by murine type 2 helper T cells (Th2) (Table 51-3; 60–64). It was first called cytokine synthesis inhibitory factor because of its ability to inhibit the production of certain cytokines by Th1. Of interest, IL-10 exhibits strong DNA and amino acid sequence homology to an open reading frame-BCRF1 in the Epstein-Barr virus (EBV) genome (1). Indeed, *BCRF1* has been called viral IL-10. The protein product of *BCRF1* (viral IL-10) exhibits properties similar to those of human IL-10. The ability of EBV to transform human B cells may be, at least in part, a ramification of the ability of viral IL-10 to stimulate B-cell proliferation.

IL-10 may have a role in the development of lymphoma through several mechanisms, including its proliferation-stimulating properties on B cells and its immunosuppressive properties that impair viral control and tumor immunosurveillance. This role has been demonstrated in a severe combined immunodeficiency (SCID) mouse model for lymphomagenesis (1). Of interest, primary B lymphoma cells from both HIV-positive and HIV-negative patients with lymphoma (NHL) secrete substantial amounts of IL-10. Indeed, several groups of investigators have evaluated the role and prognostic significance of IL-10 in patients with NHL (63,64). A series of studies has demonstrated that IL-10 levels are elevated in lymphoma patients and that high IL-10 levels correlate with prognosis, if an assay that detects both human and viral IL-10 is used. Assays that detect only human IL-10 yield no correlation with prognosis. These studies raise additional questions about the participation of EBV in lymphomagenesis.

Interleukin-11

IL-11 was originally characterized as a thrombopoietic factor but it is now known to be expressed and have activity in a multitude of other systems, including the intestine, testes, and central nervous system (Table 51-3; 65, 66). Clinically, this cytokine has been approved by the FDA for amelioration of chemotherapy-induced thrombocytopenia.

IL-11 acts as a synergistic factor with IL-3, GM-CSF, and SCF to stimulate proliferation of human primary leukemia cells, myeloid leukemia cell lines, megakaryoblastic cell lines, and erythroleukemic cell lines and to stimulate leukemic blast colony formation. IL-11 mRNA expression in leukemic cells and inhibition of leukemic cell growth by IL-11 antisense oligonucleotides suggest that IL-11 may function as an autocrine growth factor in leukemic cell lines. Although IL-11 stimulates the proliferation of murine plasmacytoma cells and murine hybridoma cells, the effect of IL-11 on the growth of human myeloma/plasmacytoma cells is controversial (65).

IL-11: Clinical Trials/Applications

A multicenter, randomized, placebo-controlled clinical trial of IL-11 versus placebo showed that IL-11 reduced the need for platelet transfusion after chemotherapy. This led to its FDA approval. Edema was the most common clinical problem associated with IL-11. Some patients developed pleural effusions, shortness of breath, and/or atrial arrhythmias (1). Lower dosages of IL-11 (10 µg/d subcutaneously) have been reported to be safe for prolonged administration and to effectively raise platelet counts in patients who had a variety of bone marrow failure states (66).

Interleukin-12

IL-12 is an NK cell stimulatory factor and is crucial to the development of Th1 cells (67). There seems to be a common pathway leading from the innate immune response to adaptive immunity—intracellular pathogens stimulate macrophages to

produce IL-12, which then promotes the development of Th1 cells from a naïve cell population. This pathway may be exploitable in the design of novel immunotherapies and vaccines (Table 51-3; 67–69). IL-12 is a potent proinflammatory molecule that is essential for resistance to bacterial, fungal, and parasitic infections. It is produced within a few hours of infection, activates NK cells, and, through its ability to induce interferon (IFN)- γ production, enhances the phagocytic and bacteriocidal activity of phagocytic cells and their ability to release proinflammatory cytokines, including IL-12 itself. IL-12 is also a key immunoregulatory molecule, especially of Th1 responses. It is produced during the early phases of infection and inflammation and sets the stage for the ensuing antigen-specific immune response, favoring differentiation and function of the Th1 T cells while inhibiting the differentiation of the Th2 T cells. IL-12 also enhances the generation of cytotoxic T cells and lymphokine-activated killer cells. IL-12 synergizes with other hematopoietic factors to promote survival and proliferation of early multipotent hematopoietic progenitor cells and lineage-committed precursor cells (1). Although IL-12 has mostly stimulatory effects on hematopoiesis in vitro, IL-12 treatment in vivo decreases bone marrow hematopoiesis and both transient anemia and neutropenia.

IL-12: Clinical Trials/Applications

IL-12 has potential for exploitation in the treatment of allergy and as an adjuvant for infectious disease therapy (69). Additionally, the ability of IL-12 to revert existing states of tolerance or anergy makes it a candidate for use in the composition of vaccines for infectious agents or tumors. Phase 1 clinical trials have begun in the last few years in oncology (with an emphasis on melanomas, renal cell carcinomas, and cutaneous T-cell lymphomas) as well as in the setting of HIV infection and chronic hepatitis B and C.

Interleukin-15

IL-15 shares biologic activities with IL-2 (Table 51-3; 70,71). Similar to IL-2, IL-15 is able to trigger both proliferation of and immunoglobulin production by normal B lymphocytes. IL-15 also stimulates the proliferation of NK cells and activated CD4⁺ and CD8⁺ T cells, and it facilitates the induction of cytolytic effector cells (such as lymphokine-activated killer cells). Finally, the numbers of CD8⁺ memory T cells are maintained in animals by a balance between the stimulatory effect of IL-15 and the suppressive effects of IL-12. IL-15 responsiveness distinguishes malignant B cells from normal B lymphocytes. In contrast to normal B lymphocytes, which require preactivation in order to proliferate in response to IL-15, leukemic cells from patients with chronic B-cell malignancies proliferate in response to IL-15 regardless of in vitro preactivation.

Interleukin-16

Cruikshank and Center first described IL-16 in 1982 (Table 51-3; 72). They found that this molecule was a lymphocyte chemoattractant factor expressed by mitogen-stimulated human

peripheral blood mononuclear cells. IL-16 has been implicated in several conditions, including asthma and granulomatous inflammation (72). It may also have antiviral effects in the context of HIV-1 (1).

Interleukin-17

Human IL-17 was originally identified by Rouvier and colleagues (Table 51-3; 73,74). Of interest, this molecule has 72% overall sequence identity at the amino acid level with open reading frame 13 of *Herpesvirus saimiri* (1). Although limited in number, studies suggest that IL-17 may be a soluble factor by which T cells induce or contribute to inflammation (74). IL-17 can also stimulate epithelial, endothelial, and fibroblastic cells and macrophages to express a variety of cytokines. The cytokines released after exposure to IL-17 seem to be cell specific. For instance, fibroblast cells produce IL-1, G-CSF, IFN- γ , IL-6, and IL-8 in response to IL-17, and macrophages produce TNF- α , IL-1 β , IL-1R α , IL-6, IL-10, and IL-12. IL-17 also exhibits indirect hematopoietic activity by enhancing the capacity of fibroblasts (through stimulation of growth factor release) to sustain the proliferation of CD34⁺ hematopoietic progenitors and their differentiation into neutrophils (74). IL-17 can also promote the maturation of dendritic cell progenitors. Because IL-17 acts to differentiate early dendritic cells, it has been implicated in host T-cell allostimulation and graft rejection (1).

Interleukin-18

IL-18 (IFN-inducing factor) was first described as a serum activity that induced IFN- γ production in mouse spleen cells (1). It is related to the IL-1 family of genes (Table 51-3; 25,75,76). IL-18 has a molecular weight of 18 to 19 kD and has homology to IL-1 (75). Like IL-1 β , IL-18 is initially synthesized as an inactive precursor molecule (pro-IL-18) lacking a signal peptide and is cleaved by ICE to yield an active molecule (1). T lymphocytes, NK cells, and macrophages are primary targets for IL-18. For example, IL-18 directly stimulates production of TNF in human blood CD4⁺ T lymphocytes and NK cells and plays an important role in promoting a long-lasting Th1 lymphocyte response to viral antigens. IL-18 does not seem to be an endogenous pyrogen but may nevertheless contribute to inflammation and fever because it is a potent inducer of TNF, chemokines, and IFN (76). In the case of IFN- γ induction, IL-18 acts as a costimulant with mitogens or IL-2. Indeed, mice deficient in ICE, the molecule that cleaves pro-IL-18 to its mature form, fail to produce IFN- γ in response to endotoxin.

Interleukin-19

IL-19 is one of the members of the human IL-10 family of cytokines (Table 51-3; 77). IL-19 shares 21% amino acid identity with IL-10, and the exon/intron structure of IL-19 is similar to that of the human IL-10 gene, comprising five exons and four introns within the coding region of the IL-19 cDNA. The expression of IL-19 mRNA can be induced in monocytes by lipopolysaccharides or GM-CSF.

Interleukin-20

IL-20 was discovered as another IL-10–related cytokine. It induces keratinocyte proliferation and causes aberrant epidermal differentiation in the skin (78). IL-20 receptor complex is described as a heterodimer of two orphan class II cytokine receptor subunits termed “IL-20R α ” and “IL-20R β ” (Table 51-3; 78–80). Recombinant IL-20 binds to its receptor on keratinocytes and stimulates a STAT3-containing signal transduction pathway (80). Experimental evidence suggests a role for IL-20 and its receptor in psoriasis, a multigenic skin disease characterized by increased keratinocyte proliferation and differentiation. Clinical applications are currently under consideration.

Interleukin-21

IL-21, a cytokine most closely related to IL-2 and IL-15 (Table 51-3; 79,81,82), is involved in the proliferation and maturation of NK cell populations from bone marrow, as well as in the proliferation of mature B-cell and T-cell populations (81). IL-21 has been implicated in the activation of innate immune responses and in the Th1 response. IL-21 also plays a critical role in regulating immunoglobulin production of B cells (82).

Interleukin-22

IL-22 was originally described as an IL-9–inducible gene and called IL-TIF (83). IL-22 activities include induction of the acute-phase response in hepatocytes. These activities are mediated through a heterodimeric receptor composed of the IL-22R subunit and the β chain of IL-10R (84). In addition to its cellular receptor, IL-22 binds to a secreted class II cytokine receptor family member that acts as a natural IL-22 antagonist (Table 51-3; 83,84). To date, IL-22 has not been applied clinically.

Interleukin-23

IL-23 is a member of the IL-6 family of cytokines and is closely related in structure to IL-12. IL-23 and IL-12 are heterodimeric cytokines that share the p40 subunit, and each has a unique second subunit, IL-23p19 and IL-12p35, respectively (Table 51-3; 85). In addition to the close structural relationship between IL-23 and IL-12, their heterodimeric receptors share the IL-12R β chain, and these cytokines have similar properties (85).

Interleukin-24

When IL-24 was discovered, it was designated as melanoma differentiation-associated gene-7 (mda-7), because it was identified by subtractive hybridization after the treatment of melanoma cells with IFN- β and mezerein, which caused their terminal differentiation and growth arrest (86). Later, it was recognized that mda-7

encodes a secreted protein that exhibits significant homology to IL-10 (Table 51-3; 79,87–91). Then, this molecule was officially designated as IL-24 (87). Human IL-24 is secreted by activated peripheral blood mononuclear cells and is the ligand for two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2 (88). IL-24 also acts as a tumor-suppressor gene, and the protein product was found to be constitutively expressed by melanocytes, nevus cells, and some primary melanomas but not metastatic lesions of melanoma (89,90). It is an example of a tumor-suppressor gene exhibiting immune stimulatory properties (91).

IL-24: Clinical Trials/Applications

Because of the tumor-suppressor characteristics of IL-24, it may potentially be used for cancer treatments in the clinic. Gene transfer of IL-24 was studied in the phase 1 setting using intratumoral injections of Ad-mda7/IL-24 (INGN 241) in 28 patients with resectable solid tumors (92). It has been reported that intratumoral administration of INGN 241 is well tolerated and induces apoptosis in a large percentage of tumor cells (92,93).

Interleukin-25

IL-25 was recently identified as a cytokine that is structurally related to IL-17 and induces IL-4, IL-5, and IL-13 gene expression (Table 51-3; 94). The induction of these cytokines results in Th2-like responses marked by increased serum IgE, IgG1, and IgA concentrations, blood eosinophilia, and epithelial cell hyperplasia. Little is known about this newly discovered cytokine besides the fact that IL-25 is derived from Th2 T cells and that it can amplify allergic-type inflammatory responses by its actions on other cells.

Interleukin-26

Subtraction hybridization coupled with representational differential analysis identified IL-26/AK155 as a gene upregulated in human T cells following infection with *Herpesvirus saimiri* (HVS; 1). It has the capacity to transform these cells in culture (Table 51-3; 79,95). The IL-26 protein has 24.7% amino acid identity and 47% amino acid similarity with human IL-10. Structural analysis revealed that IL-26 contains six helices with four highly conserved cysteine residues, which are assumed to be relevant for dimer formation as is the case with IL-10. It was determined that IL-26 mRNA is specifically overexpressed by T cells after HVS transformation.

Interleukin-27

In 2002, Pflanz and colleagues (96) described a new heterodimeric cytokine related to IL-12. This cytokine was designated IL-27. IL-27 acts together with IL-12 to trigger IFN- γ production by naïve CD4⁺ T cells (Table 51-3; 96,97). They also identified IL-27 as the ligand for TCCR/WSX-1, a novel member of the class I cytokine receptor family shown to be important for Th1

development (1). Recent studies have found that IL-27 has the ability to induce tumor-specific antitumor activity and protective immunity and that the antitumor activity is mediated mainly through CD8⁺ T cells and IFN- γ (97).

Interleukin-28 and Interleukin-29

The IL-28 family has been identified from the human genomic sequence, and the member cytokines have been designated interleukin 28A (IL-28A), IL-28B, and IL-29. These molecules are distantly related to type I IFNs and the IL-10 family. IL-28 and IL-29 are induced by viral infection and show antiviral activity. Moreover, IL-28 and IL-29 interact with a heterodimeric class II cytokine receptor that consists of IL-10R β and an orphan class II receptor chain, designated IL-28R α . This newly described cytokine family may serve as an alternative to type I IFNs in providing immunity to viral infection (Table 51-3; 98).

Interleukin-31

IL-31 has been identified as a four-helix bundle cytokine that is preferentially produced by T-helper type 2 cells. IL-31 signals through a receptor composed of IL-31 receptor A and oncostatin M receptor. Expression of IL-31 receptor A and oncostatin M receptor mRNA was induced in activated monocytes, whereas epithelial cells expressed both mRNAs constitutively (Table 51-3; 99). More specifically, the data indicated that IL-31 might be involved in promoting the dermatitis and the epithelial responses that characterize allergic and nonallergic diseases.

Interleukin-32

IL-32 is a recently discovered proinflammatory cytokine that induces TNF- α , IL-1 β , IL-6, and chemokines (Table 51-3; 100). The proinflammatory activity of IL-32 seems to take place after degradation of I κ B, leading to activation of NF- κ B as well as phosphorylation of mitogen-activated protein p38 (100). IL-32 was originally identified as a transcript, NK cell transcript 4 (NK4), the expression of which was increased in activated NK cells. It has been very recently demonstrated that NK4 is secreted from several cells upon the stimulation of some inflammatory cytokines, such as IL-18, IL-1 β , IFN- γ and IL-12 (101). The gene encoding IL-32 resides at chromosome 16

p13.3, and four mRNA transcripts resulting from mRNA splicing are presently known (100,102). Moreover, recent data suggest that IL-32 could be involved in activation-induced cell death in T cells, probably via its intracellular actions (101).

Interleukin-33

IL-33, a member of the IL-1 family, which mediates its biological effects via IL-1 receptor ST2, activates NF- κ B and MAP kinases, and it drives production of T(H)2-associated cytokines from in vitro polarized T(H)2 cells. In vivo, IL-33 induces the expression of IL-4, IL-5, and IL-13 and leads to severe pathologic changes in mucosal organs (Table 51-3; 103). Analysis of a panel of human and mouse cDNA libraries by real-time quantitative polymerase chain reaction (PCR) showed that IL-33 mRNA is broadly expressed in many tissues but is more restricted at the level of cell types. Activated dendritic cells and macrophages are the only hematopoietic cells that show low quantities of human IL-33 mRNA. Like IL-1 β and IL-18, IL-33 is produced in a precursor form and can be cleaved by caspase-1. Caspase-1, formerly termed the "IL-1 β -converting enzyme," cleaves the inactive IL-1 β and IL-18 precursors into active cytokines. Indeed, caspase-1-deficient mice fail to develop colitis, arthritis, acute renal failure, T-cell-mediated hepatitis, and metastatic melanoma. In some of these disease models, caspase-1 deficiency is due to reduced IL-1 β processing and secretion, but in other models it is due to reduced IL-18. In humans, there are several severe systemic inflammatory diseases in which an increase in functional caspase-1 activity seems to play an important role. Therefore, caspase-1 processing of precursor IL-33 may provide a therapeutic target to control allergic diseases (104).

Conclusion

Many, if not most, cytokines and their respective natural inhibitors are ubiquitously expressed and have myriad biologic properties that influence virtually every organ system. It is already apparent that these molecules may also be effective in allergic, inflammatory, and autoimmune diseases, as well as in cancer therapeutics. The emerging understanding of their role and the availability of recombinant molecules for clinical therapeutics suggest that their application is still in the evolving stages and will ultimately affect many factors of medicine.

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Interferons

Interferons (IFNs) comprise a family of secretory proteins induced in response to specific extracellular stimuli through stimulation of toll-like receptors (TLRs; [Table 52-1](#)). Acting in paracrine or autocrine modes, IFNs stimulate intra- and intercellular networks for regulating innate and acquired immunity, resistance to viral infections, and normal and tumor cell survival and death. Through high-affinity cell surface receptors IFNs stimulate genes ([Table 52-1](#)), using signaling molecules used by other cytokines, but first identified through studies of IFNs. Perturbations in these pathways can lead to overstimulation of cellular functions or can make cells resistant to a given ligand, facilitating either progression or resistance of malignancy. IFNs act on almost every cell type and through their cellular actions can be effective in inhibition of tumor emergence, progression, and for inducing regression ([Table 52-1](#)).

Studies on the mechanisms by which IFNs exert their anti-tumor activity have helped understand the role host resistance to tumor emergence and also define cellular actions of interferon-stimulated gene (ISG) products ([Table 52-1](#)). These latter proteins, of which there are more than 300 transcriptionally regulated through IFN signaling pathways ([1–4](#)), underlie not only the anti-tumor and immunoregulatory actions but also antiviral effects of IFNs. Suppression of IFNs and their stimulated gene products in and by malignant cells is emerging as an important contributor to the development of human cancer ([Table 52-2](#)). Mutation of a gene in the IFN response pathway, *RNASEL*, increases prostate cancer risk ([5–7](#)). Gene-expression profiling and cytogenetic analyses have identified decreases in ISGs in melanoma, colon, breast, and hematologic malignancies ([8–20](#)). Epigenetic and genetic silencing of IFN signaling or ISG expression of IFN-stimulated gene expression also likely influences tumor development ([18,21–24](#)). In addition to being a primary source for production of IFNs, dendritic cell maturation is also influenced by IFNs ([25–28](#)). These actions are probably the basis for effectiveness of IFNs and/or inducers in suppressing tumor emergence in carcinogen-induced tumors in preclinical models and could play a role in clinical chemoprevention ([29–33](#)).

Before development of recombinant DNA technology for protein production in prokaryotes, only limited quantities of impure IFNs were available. IFNs were, indeed, the first proteins produced by recombinant technology and not previously clinically used introduced into clinical medicine. Subsequently, a long-awaited milestone, U.S. Food and Drug Administration (FDA)

Table 52-1 Regulatory Molecules in Antitumor Mechanism of Action of Interferons

• Induction
- Toll-like receptors (TLRs)
- MyD88, RIG-I
- IRFs
• Receptors
- IFN- α R1 IFN- α R2
- IFN- γ R
• Signal transduction
- Jaks, STATs
- IRFs
• Interferon-stimulated genes (ISGs)
• Cellular effects
- Apoptosis
- Immunoregulatory
- Anti-angiogenic

approval for using defined human proteins with potent regulatory activities for treatment of human malignancy, was realized ([34,35](#)). IFNs thus became the prototypic biologic response modifier(s) in use for clinical oncologic therapy. With an emphasis on studies in human cells, this chapter discusses mechanisms of action, receptor interactions, signal transduction pathways, dysregulation of ISGs, and clinical antitumor activity.

Induction, Genes, Receptors, and Signaling

Interferon Genes, Proteins, and Their Induction

There are several types and families of interferons, all of which have antiviral effects. Current classification is based on primary

Table 52-2 Interferons in Malignant Pathogenesis

• ISGs in melanoma and other tumor cell lines
- Decreased in constitutive expression
- Increased correlates with improved prognosis
- RNase L (HPC1) mutation increases prostate cancer risk
• Murine tumor development
- Antibody to murine interferon (IFN) hastens tumor emergence
- IFNs decrease carcinogen-induced tumors
• Role in T-cell and dendritic cell maturation
• Methylation silencing of genes for IFN actions
- ISGs (XAF1)
- RASSF1A MAGE1 DAP kinase
- Thrombospondin

structures as well as target receptors. By the latter criterion, there are three types of IFNs. Type I IFNs include the multiple subtypes of the IFN- α family, IFN- β , IFN- ω , IFN- τ , IFN- κ and IFN- ϵ (35–39). The sole type II IFN is IFN- γ . The newly discovered type III IFNs are also known as IFN- λ or IL-28/29; there are three known members, $\lambda 1$ (IL-29) and $\lambda 2/3$ (IL-28 A/B; 40). The type III IFNs share structural homology and induction characteristics with type I IFNs although function through a different receptor.

The genes for type I IFN lack introns; 17 human type I IFN genes, including many encoding subspecies of IFN- α , are clustered on chromosome 9 (36–39). The corresponding 13 murine genes are clustered on chromosome 4. At the protein level, the human IFN- α subspecies share about 50% sequence identity; IFN- β is 22% and IFN- ω is 37% identical to the IFN- α . Those conserved residues are thought to mediate similar receptor recognition by these proteins. IFN- α proteins have 186 to 190 amino acid residues and contain a cleavable signal peptide resulting in secreted protein of 165 or 166 amino acids. Two Cys-Cys disulfide bonds are conserved among the proteins.

The gene encoding IFN- γ , located on human chromosome 12 and mouse chromosome 10, has three introns and encodes a protein of 146 residues function as a dimmer (41,42). The structural homology of IFN- γ with type I IFNs is minimal. NK cells are the major source of IFN- γ whereas all cell types can produce IFNs- α and IFN- β .

Type I IFNs are produced however predominantly by dendritic cells but also by T cells, monocytes, fibroblasts, and epithelial cells. The choice of specific family members that are induced depends on both the cell type and the inducing agent. Virus infection or viral gene products, such as dsRNA, ssRNA, dsDNA or viral envelope proteins, can trigger type I IFN synthesis (43–45). These viral pathogen-associated molecular patterns (PAMPs) are recognized by specific membrane-bound proteins called Toll-like receptors (TLRs) that initiate the signaling process culminating in IFN synthesis (Table 52-3). Double-stranded (ds) RNA, a common byproduct of viral replication, is recognized by TLR3, a protein present in endosomal

Table 52-3 Pathogen-Associated Macromolecular Patterns (PAMPs) as Ligands and Toll-like Receptors (TLRs) Resulting in Innate and Acquired Immunity

• Distinct macromolecules of pathogens
- Recognition of ligands by TLR
-TLR1: diacyl lipopeptides
-TLR2/6: triacyl lipopeptides (zymosan)
-TLR3: dsRNA (poly I:C)
-TLR4: LPS (paclitaxel)
-TLR5: flagellin
-TLR7/8: ssRNA/imiquimod
-TLR9: CpG DNA
- Intracellular proteins activated
-RIG-I
-PKR
-2–5 oligoadenylate synthetase
• Promote activation of innate and acquired immunity
- Synthesis of induced proteins
-Interferons (type I)
Cytokines and chemokines
- Effectors
-Dendritic cells, NK cells
-Amplification (IFN- γ , IL-2, IL-12)
- Antigen presentation

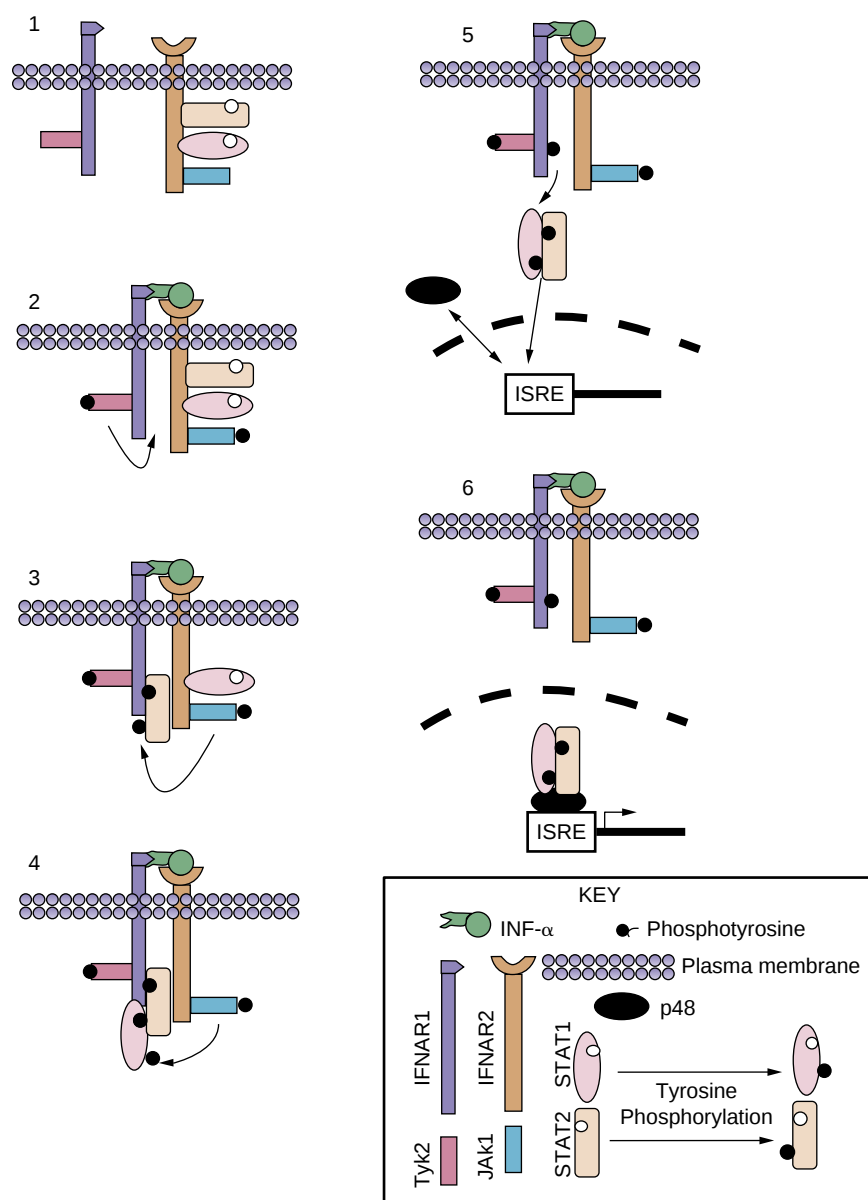
Ds, double strand; IFN, interferon; IL, interleukin; NK, natural killer; ss, single strand.

membrane (46). dsRNA can also be recognized by two cytosolic RNA-helicases, RIG-I and Mda5. Viral single-stranded (ss) RNAs are recognized by TLR7 and TLR8 and viral DNA by TLR9, all of which are also present in endosomal membrane (43,45). Different adaptor proteins connect these receptor proteins to specific protein kinases, such as TBK1 and IKK, which activate transcription factors including NF- κ B, IRF-3, IRF-7, and AP-1. For IFN- β gene induction, NF- κ B, the AP-1 complex composed of ATF2/c-jun and either IRF-3 or IRF-7 is needed. They form the enhancosome complex at the gene promoter (47,48). Synthesis of different members of the IFN- α family and IFN- β can be temporally staggered. IFN- β induces IRF-7 synthesis, which, in turn, induces transcription of IFN- $\alpha 1$ and other IFN- α genes. IFN synthesis and IFN action are, therefore, intimately linked; inhibition of IFN signaling blocks robust production of IFN- α . IRF5 can also participate in IFN- α gene induction in specific situations. Bacterial PAMPs can induce IFN synthesis using other TLRs, such as TLR4, which use different adapter proteins but activate the same transcription factors as TLR3.

IFN Receptors and Signaling

IFNs bind to cell surface receptors, which are transmembrane proteins, and trigger signaling by their cytoplasmic domains (38,39,48). The signals are transmitted to the promoters of the IFN-stimulated genes in the nucleus and their transcription is induced. Many of the same genes are also induced by dsRNA and viruses, which use different signaling pathways (46,48). Synthesis of some of the proteins that mediate IFN-signaling is induced by IFNs as well, thus eliciting positive feedback responses. In contrast, proteins, such as suppressor of cytokine signaling (SOCS), that block IFN-signaling are also induced by IFNs (49,50). The latter group of proteins terminates the signaling process so that, even in the continuous presence of IFNs, signaling is transient. The signaling pathways used by IFNs and other cytokines partially overlap. Under homeostatic conditions cross-talk among cytokines results in eventual signaling homeostasis that reflects the milieu *in vivo*.

Cells of all lineages, except mature erythrocytes, express receptors for type I and type II IFNs. Both receptors are multisubunit transmembrane glycoproteins whose extracellular domains recognize and bind the cognate IFNs. Activated cytoplasmic domains signal by binding to Jaks (Janus kinases) and STATs (signal transducers and activators of transcription; 35,38,39,48,51). Type I IFN receptors have two subunits, IFNAR1 and IFNAR2c, both which are needed for binding the ligand with high affinity (38,38,52). Their cytoplasmic domains can bind the two protein tyrosine kinases, Tyk2 and Jak 1, which phosphorylate the receptor proteins and STAT1 and STAT2 (Figure 52-1). Additional transmembrane proteins may participate in forming the fully functional receptor complex. The IFN- γ receptor has two subunits as well, IFNGR1 and IFNGR2, whose intracellular domains bind Jak1 and Jak2, respectively (41,53). The functional IFN- γ receptor consists of two molecules of each of IFN- γ , IFNGR1 and IFNGR2. The critical event in triggering



the signaling process is ligand-driven dimerization of the receptors which results in cascades of tyrosine phosphorylation.

Receptor activation upon ligand binding leads to STAT tyrosine phosphorylation (Figure 52-1). Phosphorylated STATs can form homomeric or heteromeric dimers that translocate to the nucleus and function as transcription factors by binding to specific *cis*-acting DNA elements in the regulatory regions of ISGs. The principle transcription factor, activated by type I IFNs, is ISGF3, composed of activated STAT1, STAT2, and IRF-9, a specific member of the IRF family of proteins that recognizes the DNA element ISRE (IFN-stimulated response element) in ISG promoters. Thus, for type I IFN signaling by the Jak-STAT pathway, presence of seven proteins is essential: IFNAR1, IFNAR2c, Jak1, Tyk2, STAT1, STAT2, and IRF-9. For full activation of STATs, further phosphorylation at specific serine residues is required as well. For type II IFN signaling, dimerized STAT1 is the principle transcription factor. The DNA element recognized by is the GAS (gamma activated site). In the IFN- γ signaling pathway, the two receptor-subunits, Jak1, Jak2, and STAT1 are required to activate the transcription factor, GAF, which binds to GAS. Again, additional Ser phosphorylation of STAT1 is required for the optimum function of GAF.

In addition to the major pathways, outlined previously, both type I and type II IFNs can activate other signaling pathways (51,54,55). For example, just like IFN- γ , type I IFNs can also trigger STAT1-dimer formation and gene induction by the GAS elements. Conversely, IFN- γ can activate a transcription factor that recognizes ISRE. There are also secondary signaling pathways activated by the products of some of the primary ISGs. Especially in the case of IFN- γ , several important genes, such as the major histocompatibility complex (MHC) class II genes, are induced by a secondary cascade of signaling.

Although different subspecies of IFNs- α and IFN- β signal through the Jak-STAT pathway using the same receptor, signaling pathways triggered by them may not be identical. A mutant cell line lacking Tyk2 can still respond to IFN- β and IFN- α 8, but not to IFN- α 1 and IFN- α 2. Preferential induction of the *BR1* gene by IFN- β also supports the notion of additional IFN- β -specific pathways (56). The receptors, which are glycosylated in their extracellular domains, are both necessary and sufficient for activities of IFNs with binding to IFNAR-1 accounting for the greater affinity and differential actions of IFNs- α and IFN- β (57–59). Ancillary pathways, activated by type I IFNs, are also probably triggered by additional protein kinases such as PI3 kinase (PI3K) and p38 MAPK (51,54,55).

To add to the complexity of the signaling process, several other STATs, such as STAT3 and STAT5, are activated by IFNs and they can form homo- and heterodimers to influence the nature of the overall transcription programming (51,60). Similarly, in cells of the immune system, IRF-8 can be a component of the transcription factors activated by either type of IFNs. Thus, the global impact on gene induction by IFNs is influenced by many factors, including cell lineage, exposure of the cells to other cytokines, and secondary cascades of signaling generated by genes induced in the primary response (51,55). IFN- λ proteins signal through a different receptor that is composed of a unique IFN-LR1 subunit

and the IL-10R2 chain of IL-10, IL-22, and IL-26 receptors. Like other type I IFNs, IFN- λ uses the proteins Jak1, Tyk2, STAT1, and STAT2 for signaling, but additional pathways are probably activated as well. Another major difference is in the expression profile of the receptors. Whereas IFNAR1 and IFNAR2 are ubiquitously expressed, expression of IFN-LR1 may have a relatively limited distribution on dendritic cells (40,61).

Inhibition of Action and Molecular Oncogenesis

The mechanisms involved in downregulation and termination of IFN signaling are multifaceted. Receptors and activated STATs are dephosphorylated, particularly by SHP-1 and SHP-2 but also other tyrosine phosphatases; inhibition of these can prolong signaling and potentiate antitumor activities of IFNs (62–64). As mentioned previously, activated Jaks are inhibited also by the SOCS family of proteins, themselves induced by IFNs. Several viral oncoproteins can also block IFN-signaling, often by interfering with the function of ISGs or ISGF-3 (65,66).

Signaling events in the JAK/STAT pathway also can have a critical role in malignant pathogenesis. Loss of function of a STAT-modifying phosphatase resulted in melanocytic neoplasms in *Drosophila* (67). Mice lacking IRF-8 develop a disease similar to chronic myelogenous leukemia (CML). Overexpression of IRF-8 in Bcr-Abl-transformed cells inhibited leukemogenesis as well as resistance to the tyrosine kinase inhibitor, imatinib: this correlated with reduced bcl-2 expression (68). Furthermore, aberrant JAK2 expression, part of a protein complex driven by the Bcr-Abl translocation in CML, could be a target for therapy of imatinib-resistant CML (69). Since the activity of IFN- α 2 in CML has never been well understood on a molecular basis, tyk1 and JAK2 stimulation by IFN- α 2 may be providing a lateral, suppressive shift in JAK2 activity with subsequent inhibition of proliferation.

Further evidence suggesting a direct role for IFNs on tumor cell growth and viability through inhibition of proliferation or apoptosis has emerged using mouse tumor model systems. Treatment with human IFN- α or IFN- β of nude mice implanted with human tumor cells effectively controlled tumor growth and has promoted tumor cell apoptosis (70,71). Alteration of tumor phenotype by reversal of epigenetic silencing of gene expression made an IFN-resistant mouse tumor sensitive (72). Since mouse cells do not respond to the human IFNs, these antitumor effects were clearly direct. Furthermore, progression of a *ras*-transformed cell line could be reversed both *in vitro* and *in vivo* by IFN- β with or without retinoic acid (73).

In experimental systems, equivalent antitumor effectiveness of IFNs *in vivo* has been identified for tumor cells sensitive or resistant to antiproliferative effects of IFNs *in vitro* (74,75). Additional evidence, supporting a role for host immune effector cell response to IFNs, comes from studies in which mice implanted with Friend leukemia, a syngeneic tumor or with human prostate and HeLa tumor xenografts received neutralizing antibody to murine IFN (76). These mice, in the absence of exogenous IFN, had enhanced tumor growth and transplantability, suggesting that neutralization of endogenous IFN removes aspects of host defense to tumor.

Other studies have also highlighted the potential importance of immune cell regulation in the antitumor effects of type I IFNs. *STAT1* knockout mice implanted with IFN-responsive tumors did not have enhanced survival in response to exogenous IFN- α (77), whereas wild-type (*STAT1*^{+/+}) animals implanted with *STAT1*-null tumor cells were able to mount an effective antitumor response following IFNs (78), suggesting that the antitumor response to IFN relies more on the effects on host tissues than the tumor itself. Cell-depletion studies were performed to assess the types of immune cells involved; these highlighted the critical importance of NK cells. However, the clinical observation that autoimmune events were associated with protection from recurrence of melanoma in patient treated with IFN- $\alpha 2$ has suggested indirectly the possible importance of T-cell augmentation for antitumor effects (79), possibly as a result of depletion of activity regulatory T cells.

However, regardless of the cellular mechanisms of antitumor action, which may differ between tumor types, underlying the immunoregulatory, antiproliferative/prodeath, and anti-angiogenic effects of IFNs are the ISGs, which are regulated at the level of transcription. Functions of many of these ISGs are not yet well understood; many were identified as differentially expressed mRNAs or enzymes after treatment of cells with IFNs and more recently on expression arrays. Although overlap in function certainly exists, we will relate the actions of the most studied protein products of ISGs to primary cellular activities relevant to antitumor effects in what follows.

Mechanisms of Antitumor Action and Induced Genes

Antigrowth/Apoptosis Effects

Because IFNs possess antigrowth and pro-apoptotic activities in vitro, ISG products that may mediate this action have been a focus of assessment of molecular actions (Table 52-4). Unlike apoptosis induced by camptothecin through stimulation of the extrinsic apoptotic cascade, programmed cell death through the intrinsic cascade in response to IFNs is a late effect requiring 48 to 72 hours. Underlying this latency is probably the requirement for induction of ISGs, many of which have been identified by expression profiling (1-4). A role for the ISG product, Apo2L/TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) in IFN-induced apoptosis in myeloma,

melanoma, T cells, B cells, hepatoma, and lymphoma cells has been identified (80-82). Coculture of cells with IFN and neutralizing TRAIL antibodies or TRAIL decoy receptors can inhibit IFN-induced apoptosis. In melanoma, renal carcinoma and myeloma cells, if both TRAIL and XAF1 were not induced by IFNs, no apoptosis resulted. IFN- β induced Apo2L/TRAIL in melanoma and other cells more potently than did IFN- $\alpha 2$ (81). Combination treatment with IFN and RA synergistically induced Apo2L/TRAIL, which may explain synergistic anticancer effects (73,83). Apo2L/TRAIL induction in breast cancer cells by IFN and retinoic acid was mediated through IRF-1 activation of the Apo2L/TRAIL promoter (83).

Other ISG protein products are, however, also needed to sensitize cells to the effects of Apo2L/TRAIL. Pretreatment with IFNs, particularly IFN- β , can enhance apoptosis from recombinant Apo2L/TRAIL or its receptor agonists, even in cells otherwise resistant to Apo2L/TRAIL (81). ISGs, such as the p200 family, PML, Fas L, and XAF1 (13,84-90), among others, also have substantial effects on cell proliferation and viability. XAF1 was correlated with the ability of cells to respond to the pro-apoptotic effects of Apo2L/TRAIL (86). Through interactions with p53 and the inhibitor of apoptosis, XIAP (86,87), XAF1 may allow Apo2L/TRAIL to fully activate downstream caspases. Another ISG product, IRF1, suppressed another anti-apoptotic protein, survivin (88).

The nuclear protein, PML, may be involved in IFN-induced Apo2L/TRAIL expression in myeloma cells (89,90). PML, which acts as a tumor suppressor, has been identified as contributing to cellular apoptotic responses. Mice deficient in PML are largely resistant to IFN-induced apoptosis. Nuclear bodies (NBs), multiprotein complexes in the nucleus associated with acute promyelocytic leukemia and AIDS, are made up of the PML protein, sp100, sp140, sp110, and the exonuclease ISG-20, all of which are ISG products (90). In contrast to the pro-apoptotic actions of products of many ISGs, G1P3 (ISG6-16) inhibited apoptosis in gastric carcinoma and myeloma (91,92).

In CML and multiple myeloma cells induction by IFN- α of the death receptor Fas/CD95 resulted in apoptosis through recruitment of FADD (Fas-associated death domain) and subsequent activation of caspase-8/FLICE (93). Intralesional administration of IFN- α into basal cell carcinomas increased Fas expression and led to regression (94). Similarly IFN- γ increased susceptibility of melanoma cells to apoptosis by Fas activators and breast carcinoma cells to doxorubicin (95,96). The latter drug also induced *STAT1* (96); *STAT1* may also be induced by paclitaxel since among other genes induced in a regressing ovarian carcinoma xenograft were the ISGs, *G1P3*, *IFI16*, *IFI27*, *IFITM1*, and *ISG15* (97).

Identified initially as an IFN- γ -induced pro-apoptotic gene, death-associated protein kinases (DAPKs) are serine threonine kinases with ankyrin and death domains that induce caspase-independent cell death (98). Reduced expression correlated with malignant progression whereas restoration of expression led to apoptosis of mouse lung tumors (99). Expression of DAPK can be suppressed in human malignancies, at least in part by epigenetic silencing (21), further supporting the role of DAPKs as tumor suppressors. DAPK7, also called ZIP kinase, phosphorylated

Table 52-4 Interferon-Regulated Proteins Contributing to Apoptosis

• Apo2L/TRAIL	• Fas Ligand
• IRF-1	• XAF-1
• PML	• DAPK
• RNase L	• Protein kinase R
• p56 family proteins	• IFI-16, AIM-2 (p200 family proteins)
• IHPK2	

MDM2 and p21^{WAF1} with resultant prolongation of p21^{WAF1} half-life, providing another link of p53 and IFN pathways (100).

In addition to TRAIL and other apoptosis pathway ISG products, 2',5'-oligoadenylate synthetase (OAS), PKR and Mx are important in tumor pathogenesis. The OAS genes function in an antiviral and antitumor pathway known as the OAS-RNase L system (Figure 52-2). In humans there are three functional OAS genes (OAS1–3), resulting in 8 to 10 OAS isoforms due to alternative mRNA splicing (101). When stimulated by dsRNA, OAS proteins produce a series of short 5'-phosphorylated, 2',5'-linked oligoadenylates collectively referred to as 2–5A [$p \times 5'A(2'p5'A)_n$; $\times = 1-3$; $n > 2$] from ATP. Because dsRNA is a frequent viral PAMP, 2–5A often accumulates in IFN-treated and viral-infected cells (102,103).

The only well-established function of 2–5A is activation of the latent endoribonuclease, RNase L. Accordingly, *RNase L*^{−/−} mice have enhanced susceptibility to infections by a range of different RNA viruses. Ultimately, sustained activation of RNase L triggers a mitochondrial pathway of apoptosis that eliminates virus-infected cells (104–106). Genetic lesions in RNase L impair this apoptotic response, which has raised interest in the possibility that such mutations might also contribute to malignancy (Figure 52-2; 107,108). In this regard, the hereditary prostate cancer 1 (*HPC1*) gene maps to the RNase L gene (*RNASEL*) and is implicated in controlling apoptosis of prostate cancer cells (5). In a large, controlled sib-pair study, an RNase L variant (R462Q) with decreased enzymatic activity was implicated in up to 13% of unselected prostate cancer cases (5). However, although several case-controlled genetic and epidemiologic studies support the involvement of *RNASEL* (and notably the R462Q variant) in prostate cancer etiology (5–7), others do not (109–111), suggesting that either population differences or environmental factors such as infections may modulate the impact of *RNASEL* on prostatic carcinogenesis.

A novel retrovirus (XMRV) has been identified in the prostate tumor-bearing tissues, almost exclusively in men with the homozygous, reduced activity variant of RNase L (R462Q; 112,113). XMRV was localized within human prostate tissues

to fibroblasts and hematopoietic elements adjacent to carcinoma. Although it is unknown if XMRV contributes to prostate cancer, such infections could possibly induce paracrine growth factors or pro-inflammatory cytokines that modify the tumor microenvironment. Other findings suggest a wider tumor-suppressor role of RNase L beyond prostate cancer. One such study suggested that RNase L E265X and R462Q variants may contribute to familial and sporadic pancreatic cancers and a R462Q variant of *RNase L* correlated with earlier age of onset of hereditary non-polyposis colorectal cancer (114,115).

PKR, an IFN-inducible, dsRNA-dependent serine/threonine protein kinase that inhibits protein synthesis initiation, is activated in a process involving dsRNA, dimerization and autophosphorylation (116). In addition, a cellular protein PACT directly activates PKR in the absence of dsRNA (117). Phosphorylation by PKR of the protein synthesis initiation factor, eIF2- α causes an inactive complex to form between eIF2-GDP and the recycling factor, eIF2B. These events result in global inhibition of protein synthesis rates and suppression of viral replication; many viruses evade innate immunity by inhibiting PKR function (65,66).

PKR is a 551-amino acid protein in which the N-terminal region contains two dsRNA binding motifs while the catalytic domain is present in the C-terminal half (118,119). PKR mediates signal transduction and apoptotic and tumorigenic responses (120,121). The antitumor actions of both RNase L and PKR could involve induction of apoptosis (Figure 52-2). Mouse fibroblasts lacking PKR are resistant to apoptosis induced by TNF, dsRNA or lipopolysaccharide (121). Phosphorylation of eIF2 α by PKR is required for the apoptotic responses to dsRNA, TNF- α , or serum deprivation (122). Implantation into mice of NIH3T3 fibroblasts expressing a catalytically inactive, dominant negative PKR led to tumorigenesis (123). Deficiencies in PKR activity, but not protein, have been observed in B-cell chronic lymphocytic leukemia (CLL; 124). Because PKR is a key point of regulation in the protein synthetic machinery and can cause apoptosis, antitumor strategies for selectively activating PKR in cancer cells have been explored.

Like many of the other ISGs discussed in this chapter, ISGp56 gene (also known as *IFIT1*) is strongly induced (50–100 times) by type I IFNs (125). These proteins (including related p54, p56, and p60) contain multiple tetratricopeptide repeat (TPR) motifs that mediate protein–protein interactions. Although, the human p56 and the mouse p56 proteins have only 50% sequence identity, their six TPR motifs are positioned very similarly along the linear sequences. The same is true about three of four TPR motifs of the human and the mouse p54 proteins. A major property of the p56 and the p54 proteins is inhibition of initiation of protein synthesis (125,126). This inhibition is mediated by binding to specific subunits of the translation initiation factor eIF3; human p56 binds to the “e” subunit whereas p54 binds to both subunits. Binding of these proteins to the “e” subunit blocks the ability of eIF3 to stabilize the ternary complex of eIF2, GTP and Met tRNA. In both cases, CAP-dependent translation initiation is severely blocked.

In hepatitis C virus (HCV)-infected cells, p56 was associated with polysomes that were translating HCV mRNA; in cells expressing p56, viral replication was strongly inhibited (127).

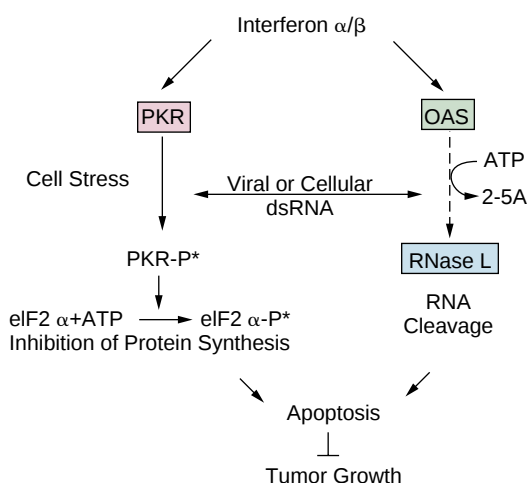


FIGURE 52-2 Interferon-regulated activation of 2–5A and PKR pathways and their mechanisms of action.

Whether this effect on p56 influences oncogenic effects of HCV has not been determined but a subunit of eIF3, to which p56 bound, was identical to *Int-6*, a gene whose disruption by the integration of the mouse mammary tumor virus gene caused mouse mammary carcinoma (128). The cellular function of the eIF3e/*Int-6* protein in this context could be different from translation initiation, because the protein was present not only in the cytoplasm as a component of eIF3, but also in the nucleus.

The p200 family of ISG protein products, IFI-16, myeloid cell nuclear differentiation antigen (MNDA), and absent in melanoma 2 (AIM2), share a common 200-amino acid domain important for protein–protein interaction and also play a significant role in cellular growth regulation and differentiation (12,129–133). They influence function of the tumor-suppressor genes Rb, p53, and the transcription factor E2F. Inhibition of proliferation of these p200 gene products resulted in loss of the transformed phenotype and decreased tumor formation in mouse models. p200 proteins may also induce the CDK inhibitor, p21^{WAF1}. In endothelial cells migration and proliferation of endothelial cells was suppressed by IFI16 (132).

Inositol hexakisphosphate kinase2 (IHPK2) was identified as a regulator of IFN-induced death in ovarian cancer cells by an antisense technical knockout approach (134). It is located on chromosome 3p21, a region frequently affected by loss of heterozygosity and chromosomal rearrangements in human cancer. Overexpression of IHPK2 sensitized tumor cell lines to apoptosis in response to a variety of chemotherapeutics and ionizing radiation, suggesting a role in a central apoptotic pathway (135).

Immune Effects

The antitumor activity of IFNs in vivo may be mediated by activation of immune effector cells, increased antigen processing, and enhanced immunogenicity of tumor cells (Table 52-5). Plasmacytoid dendritic cells have been identified as the cells that secrete high levels of IFNs- α and IFN- β in response to stimuli, a process partially regulated by osteopontin (25–28,136–139), have been found associated with the T-cell zone of lymphatic tissue and have been identified in tumors of various types. Production of IFNs furthers dendritic cell maturation and induction of T cells into a Th1 pathway.

IFNs have enhanced activity of cytotoxic and helper T cells, natural killer (NK) cells, macrophages, and dendritic cells. Cytotoxic T-cell expansion can be promoted by IFN- α through

up-regulating IL-2 (140,141). IFNs have activated NK cells and macrophages in vitro and in vivo (142). Furthermore, IFNs induced expression of Apo2L/TRAIL on immune effector cell surfaces, sensitizing tumor cells to T cell and NK cell-mediated cytotoxicity (143,144).

In addition to stimulating immune effector cells, IFNs have up-regulated MHC antigens facilitating activation of CD8⁺ cytotoxic T cells (145–147). IFNs have not only up-regulated transcription of MHC class I genes but have also coordinately induced expression of additional proteins required for the surface expression of the mature MHC class I complex. This complex contains the MHC class I polypeptide, β_2 -microglobulin, and an eight-to-ten-amino acid antigenic peptide bound to the MHC polypeptide. As nascent class I molecules are synthesized, they associate with β_2 -microglobulin, also an ISG, in the endoplasmic reticulum for transport to the cell surface. Augmentation of HLA class I-associated β_2 -microglobulin has been identified upon has treatment of patients with IFN- α 2 or IFN- β (148,149). Generation of antigenic peptides for loading onto class I molecules occurs in the proteasome. The three subunits, low-molecular-weight protein-2 (LMP-2), LMP-7, and LMP-10, which make up the proteasome, and the transporter associated with antigen processing (TAP) are all ISGs (14,17,150). TAP transports the processed peptides from cytosol to the ER for loading onto class I molecules. The MHC class II transactivator factor (CIITA), considered the master regulator of MHC class II expression, is induced by IFN- α . In addition, three cathepsins, thought to be partly responsible for peptide antigen processing and loading onto MHC class II proteins, were also up-regulated by IFN- α (151). Since a cascade of immunoregulatory cytokines including IL-12 and IFN- α can be increased by IFN- β , substantial amplification of immune effector cell function may result administration of IFNs.

Amplification of innate and specific immune responses can result from IFNs. IFNs can induce tumor-associated antigens, carcinoembryonic antigen, and TAG-72 on the surface of tumor cells both in vitro and in vivo (152). This increase could be beneficial either for innate or adoptive immunotherapies targeting these tumor antigens. Both in vitro and in patients IFNs have increased expression of a number of chemokines that function as chemoattractants (153–155). IFN-induced chemokines include CCL5 (RANTES), CSCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1a), CXCL9 (MIG), and CXCL11 (I-TAC). STAT1 induction of chemokines CXCL9, CXCL10, CXCL11, and CXCL16 controlled recruitment of protective, antigen specific Th1 cells into peripheral tissues (156). These proteins are chemoattractive to both lymphocytes and monocytes and play a crucial role in recruiting these cells into tissues.

ISG products such as phospholipid scramblase 1 (PLSCR1), like tumor-associated antigens localized to the plasma membrane, may play a role in providing macrophages with a signal for engulfment after tumor cell apoptosis (157–159). PLSCR1 may increase expression of a subset of ISGs influencing apoptosis and other effects of IFNs (157–159). Ovarian cancer cells overexpressing PLSCR1 grew more slowly in nude mice compared with vector-transfected cells, and resultant tumors were infiltrated by neutrophils and macrophages. Thus, by inducing PLSCR1, IFNs may facilitate tumor cell phagocytosis by macrophages.

Table 52-5 Interferon-Regulated Proteins Contributing to Immune Response

• MHC class I	• MHC class II
• LMP-2, LMP-7	• TAP
• CEA	• TAG-72
• CCL chemokines	• CXC and CXCL chemokines
• Phospholipid scramblase	• ISG-15

MHC, major histocompatibility complex.

ISG-15, a secreted protein induced by IFN- α and IFN- β , induced IFN- α synthesis by T cells and proliferation of NK and lymphokine-activated killer (LAK) cells (160). Four of the top 36 expressed genes in an expression array of melanoma cells (ISG 15, USP18, UBE1L, UBE2L6) constitute part of a cascade of ubiquitin-related ISGs (3). The unique protein products of these genes can conjugate intracellular proteins that, once induced, influence action and cell function of IFNs (161,162). Despite their high level of expression after IFNs, how these proteins influence cell function is an area of only limited understanding. However, they influence both innate immunity in malignant cells and cellular signaling (163–166). Melanoma cells producing high levels of ISG-15 were those able to induce E-cadherin expression on dendritic cells. Conjugation of ISG15 to important components of the IFN-signaling pathway (STAT1 and Jak1) and other signaling pathways (ERK 1, phospholipase C, and heat shock proteins) suggest that targeted regulation of ISG 15 family molecules could have profound effects not only on IFNs but also on host response to virus and cancer.

Angiogenesis Inhibition

IFNs can inhibit angiogenesis both by altering the stimulus of tumor cells and by inhibiting endothelial cells through both increase and decrease of mediating proteins (Table 52-6). Following IFNs, vessels underwent coagulation necrosis (167). Inhibition of angiogenesis by IFNs occurred before antiproliferative effects on tumor cells and have been identified in vivo within 24 hours of tumor cell inoculation (168). IFN-sensitive and -resistant bladder carcinoma cells with IFN- α in mice have identified reduction in tumor cell growth in the IFN-sensitive cells by directly regulating the expression of basic fibroblast growth factor (bFGF), an angiogenic growth factor (169). Suppression of bFGF correlated with reduced vascularization and tumor growth (169). Knock-out studies confirmed signaling through STAT1 as necessary for reduction by IFNs of bFGF signaling. Compared with wild-type mice, IFNAR receptor knockout mice have increased angiogenesis and tumorigenesis (170). Endogenous type I IFN signaling may play a role as a negative regulator of angiogenesis, keeping the “angiogenic switch” in the off position.

In addition to its action on bFGF, IFNs have inhibited angiogenesis by acting on other angiogenesis mediators. IFNs inhibited VEGF mRNA and protein expression in neuroendocrine tumors by regulating VEGF promoter activity (171). IL-8, a mediator of angiogenesis, was inhibited in vitro and in vivo by IFN- α 2b and IFN- β (172,173). Other angiogenesis inhibitory members of the chemokine family which lack the ELR binding

motif, CXCL9, CXCL10, and CXCL11 are also IFN-stimulated genes (174,175). Several GTPases, a family of proteins that function as molecular switches in signal transduction, are induced by IFNs. These include the Mx proteins and a family of guanylate-binding proteins (GBPs). GTPases have a distinct hydrolysis profile, since they bind GTP, GDP, and GMP with similar affinity and can hydrolyze GTP to GDP or GMP (176). In endothelial cells hGBP1 functioned as an inflammatory response factor inhibiting endothelial cell proliferation and angiogenesis in part through matrix metalloproteases (177,178). Thus, induction of ISGs that function as angiostatic inhibitors, coupled with secondary down-regulation factors, may contribute to inhibition of angiogenesis by IFNs (179). IFNs have inhibited tumor vascularization at doses that are well within those that are therapeutically achievable. Clinically, IFN- α 2b has proven effective in the treatment of infantile hemangiomas, hemangioblastomas, giant cell tumor of the mandible, and Kaposi sarcoma (179–182).

Antitumor Effects in Humans

Because of their clinical effectiveness in reducing tumor cell mass, limiting virus replication, controlling disease symptoms, and prolonging survival, IFNs are now licensed in more than 50 countries for treatment of various viral, malignant, and immune disorders; market sales approach \$4 billion. IFNs as single agents can induce clinical regression in almost a dozen hematopoietic malignancies or solid tumors (reviewed with primary references in more [182] and in overview references below). In CML, melanoma, renal cell carcinoma, bladder carcinoma, Kaposi sarcoma, hairy cell leukemia, lymphomas, myeloma, polycythemia vera, locally advanced basal cell carcinoma, and essential thrombocythemia, IFNs have had therapeutic value.

For example, use of IFN- α 2 for hairy cell leukemia and for CML resulted in a gradual decrease in bone marrow infiltration with malignant cells as well as a normalization of peripheral hematologic parameters (183–188). In CML, in addition to reduction in leukemic cell mass, a decrease resulted in cells with the abnormal, activated bcr-abl kinase. The median survival for all patients with CML treated with IFN- α 2 has been approximately 6 years, but over 90% of those with complete cytogenetic response were in remission at 10 years. Frequency of cytogenetic response and survival were further enhanced by adding cytosine arabinoside to IFN- α 2. However, the survival advantage for IFN- α 2, when compared with chemotherapy for CML, has now been exceeded by the substantial effectiveness of the targeted inhibitor of the activated bcr-abl kinase, imatinib, and other newer tyrosine kinase inhibitors. Similar progress has occurred in treatment of other hematologic malignancies with newer and targeted biological therapeutics, largely eliminating common use of IFN- α 2.

Curative elimination of metastatic malignancies can result when systemic therapies are given to eliminate micrometastases for patients at highest risk for recurrence after surgical removal of a primary. Effectiveness of IFNs as adjuvant therapy after surgery of murine tumors has been demonstrated (189). The pioneering studies of IFNs in therapy for osteosarcoma involved this

Table 52-6 Interferon-Regulated Proteins Contributing to Angiogenesis Inhibition

• bFGF decrease	• VEGF decrease
• GBP1	• IL-8 decrease
• CXCL-9, CXCL-10, CXCL-11	• tryptophanyl-tRNA synthetase

IL, interleukin; VEGF, vascular endothelial growth factor.

clinical approach (190). In this instance, IFN- α 2 enhanced benefit of surgery and resulted in effects equivalent to chemotherapy. To rigorously define the role of pegylated IFN- α 2 after surgery and chemotherapy for primary osteosarcoma, a randomized international trial involving cooperative groups in Europe and the United States is ongoing to confirm this clinical effect.

Elimination of micrometastases was the basis for evaluation of IFN- α 2b for patients at high risk for recurrence of melanoma: Those patients marked by deeply invasive primary melanomas or nodal metastases. The initial positive findings have been largely further validated by a combined analysis of subsequent U.S. trials of high dose IFN- α 2b for 1 year and by two meta-analyses (191,192). An ongoing trial in the United States is evaluating the addition of a three-drug chemotherapy regimen and IL-2 to IFN- α 2 alone in an intensive 3-month program. An innovative trial in Europe is evaluating pegylated IFN- α 2 for a period of 5 years with dose adjusted for the side effect of fatigue to maintain full activity level.

Building upon the therapeutic activity of IFNs in phase 2 trials in both solid tumors and hematologic malignancies, international phase 3 trials have been conducted with substantial survival impact identified in metastatic renal carcinoma, resection of a primary followed by IFN- α 2, extended patient survival in each trial cohort, and in a combined analysis (193). However, like CML, the orally active, targeted tyrosine kinase inhibitors have changed the natural history of renal carcinoma, extending survival in metastatic disease more than the injectable IFN- α 2. In follicular lymphomas in combination with chemotherapy, even relatively limited amounts of IFN- α 2 can prolong survival (194).

As inducers of IFNs, TLR agonists, imiquimod, and the phosphorothioate oligoribonucleotide CpG7909, an agonist for TLR9, have immunomodulatory effects (195,196). Imiquimod has proven effective topically for both venereal warts caused by papilloma viruses and for basal cell carcinomas (197,198). CpG7909 has entered phase 3 trials for patients with non-small cell bronchogenic carcinoma.

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Perspective

Introduction and clinical use of IFNs are one of the major advances in oncology over the past three decades. The 1980s saw the clinical introduction of these high purified pharmaceuticals as the first products of biotechnology for treatment of cancer. The 1990s were marked by an expansion in clinical use and a clearer understanding of the molecular events that influence the biologic actions. The first decade of the 21st century has been marked by an application of this knowledge in order to further understand mechanisms of action of IFNs and as part of the innate immune response mediated through TLR activation.

IFNs provide fundamental cellular defense mechanisms against viral infections and cancer and are thus critically important to the health of animals and humans. In the innate immune response, IFNs are the principle cytokine that blocks viral replication through the action of specific IFN stimulated genes reviewed in this chapter. Because all biologic effects of IFNs are mediated through the action of IFN-stimulated genes, understanding the functions of these genes may lead to more efficacious cancer and antiviral therapeutics. For example, certain IFN-regulated proteins, such as OAS, RNase L, and PKR, exist in either a latent inactive or active states. Future drugs that may act as a molecular switch for these proteins might be expected to have potent antitumor and/or antiviral effects.

Many important questions remain unanswered. What specific roles do the multiple isoforms of IFN- α play in host defenses? How do STAT proteins regulate apoptosis, cell growth, and immune responses? What are the functions of the still many uncharacterized ISGs? Which of the ISGs are most important for the antitumor effects? What mechanisms cause the difficult spectrum of clinical side effects of fatigue and anorexia? What causes resistance to IFNs in cancer? Can effective oral systemic inducers be identified? Answers to these questions will continue to stimulate future advances.

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Cancer and the Cellular Immune Response

The distinguishing features of the immune system are its abilities to distinguish self from non-self, to recognize and respond to a myriad of foreign molecules (antigens) with exquisite specificity, to remember previously encountered antigens and quickly mobilize an expanded response, and to scan the entire body continuously for antigens. These properties make the immune system ideal for defending the host against developing and recurrent cancers and nascent metastatic tumor deposits, at least in theory. In spite of its promise, however, the application of immunologic approaches to the cancer problem has not yet yielded the dramatic results long anticipated by immunologists (1–3). The major obstacle to using the immune system and its various components to prevent or treat human cancer has been a lack of understanding of the fundamental mechanisms that govern the immune response. These include the mechanisms by which cells of the immune system recognize antigens, expand and differentiate, find and destroy aberrant cells and pathogens, and die or become quiescent when no longer needed. The inability to direct these processes toward the destruction of cancer cells in the body in a consistent and effective way has thwarted most of the attempts at cancer immunotherapy to date. However, there are clear instances in which immunologic approaches have brought significant benefit to individual cancer patients (1–3). These cases sustain the belief that the immune system still has much to offer in controlling cancer, if only we could control and direct its destructive powers.

Very recently, major advances have been made in understanding how cells of the immune system become activated upon encountering an antigen and how they communicate with their external environment and with each other. Some of these advances are a direct result of the Human Genome Project, which has enabled the discovery of previously unknown genes coding for families of cytokines and receptors involved in regulating inflammatory and immune responses (4). Others are derived from insights gained from studies of how the immune system defends the host against microbial pathogens. The normal host has various mechanisms that help rid it of invading pathogens (5). Collectively, these mechanisms are called innate or natural immunity, and they require no previous contact with the pathogen to be active. Acquired immunity, on the other hand, is an immune response triggered by an encounter with a foreign cell or molecule and represents an amplification process that increases the number of lymphocytes able to recognize and respond to an invader. Until

recently, the means by which innate immune mechanisms distinguish pathogens from self were unknown. Recent studies demonstrate that the innate immune system recognizes many lethal microbial pathogens by means of pattern recognition receptors (Toll-like receptors, TLRs) on antigen-presenting cells (6–9). These receptors recognize common molecular patterns shared among bacteria or viruses but not present on normal or cancerous host cells, such as lipoprotein, peptidoglycan, lipopolysaccharide, lipotechoic acid, bacterial DNA, and viral double-stranded RNA. The triggering of pattern recognition receptors by such molecules has three important effects on the antigen-presenting cells of the immune system: (1) Expression of stable and high levels of pathogen-derived peptide-MHC complexes on the cell surface, which will trigger T-cell receptors; (2) expression of high levels of co-stimulatory molecules, such as CD80 and CD86 that prime and activate antigen-specific T-cells; and (3) secretion of a large number of proinflammatory cytokines, such as IL-1, IL-6, IL-12, TNF- α , GM-CSF, and type 1 interferon (6–9). The proinflammatory cytokines further activate antigen-presenting cells, directly activate the innate killer cells of the immune system (NK cells and macrophages), and promote T-cell differentiation into antigen-specific effector Th1 cells or cytotoxic T-cells that mediate acquired immunity. These new findings explain the mechanism of action of bacterial adjuvants, which have long been used in animal models to enhance immunization with protein and peptide antigens. They also suggest that the relative inefficiency of immunization with tumor antigens may be due, in part, to failure of the vaccines to stimulate the innate arm of the immune system, which is required for maximal amplification of antigen-specific, T-cell-mediated (acquired) immune responses. Thus, developing approaches to trigger innate immunity in concert with more traditional approaches to stimulate acquired immunity represents a promising new strategy for cancer immunotherapy.

Other important advances concern the biology and activities of dendritic cells (DCs; 10–12). These are a heterogeneous group of cells involved in antigen detection, capture, processing, and presentation to T-cells. DCs are the major cell type expressing germ-line-encoded immunoreceptors (TLRs, C-type lectin-like receptors, Ig-like molecules), and they are at the forefront of host defense mechanisms against microbial pathogens and altered self-molecules, such as those encountered in virus-infected cells and autoimmune and antitumor responses (10–12).

Innate Recognition of Microbial Pathogens by Toll-Like Receptors

Recognition of Pathogen-Associated Molecular Patterns

An essential role of our immune system is to sense and protect us from infection by pathogenic microorganisms and establish tolerance to self-antigens. One of the most important mechanisms that the immune system distinguishes “infectious non-self” from “self” is the development of recognition of “pathogen-associated molecular patterns” (PAMPs; 5). This PAMPs recognition system was first identified as Toll in *Drosophila*, an essential molecule for embryonic patterning, as well as for antifungal immunity (13). The first mammalian homologous of Toll was identified as Toll-like receptor 4 (TLR-4), which was shown to recognize lipopolysaccharides (LPSs) from gram-negative bacteria. Signaling TLR-4 triggers antigen-presenting cells to up-regulate MHC-class I/class II molecules and costimulatory molecules, as well as secrete proinflammatory cytokines, and therefore enables DCs to activate T-cell-mediated adaptive immune responses (14,15). Up to now ten TLR family members were found in human and 12 TLR family members were found in mice, which recognize the pathogen-associated molecular patterns, including proteins, lipids, and nuclear acid (6–9).

TLR Structure

TLRs are type 1 transmembrane proteins. The extracellular portion of TLRs contains the leucine-rich repeats, which is essential for ligand-binding. The intracellular portion shares a common structure with IL-1 receptor, called Toll/IL-1 receptor homologous (TIR) domain, which is critical for signal transduction. The TLR genes are dispersed throughout the genome, with *TLR1* and *TLR6* genes on chromosome 4p14, *TLR2* and *TLR3* on 4q33-q35, *TLR4* on 9q32-q33, *TLR5* on 1q33.3-q42, *TLR7* and *TLR8* on Xq22, and *TLR9* on 3q21.3 (6–8).

TLR Subdivision and Ligands

The phylogenetic analyses of the amino acid sequence and structure of TLRs, in combination with the analyses of the TLR ligand binding suggest that TLRs are evolved along two major pathways (Figures 53-1 and 53-2; 6–8):

1. Recognition of nuclear acid: TLR7 and TLR8 recognize single-stranded viral RNA; and TLR9 recognize double-stranded bacterial and viral DNA. TLR3 recognize double-stranded viral RNA.
2. Recognition of lipid and lipoprotein: TLR4 recognize LPS, TLR1, TLR2 and TLR6 recognize bacterial lipoprotein; and TLR5 recognize bacterial flagellin.

TLR Signal Transduction

Currently, three major pathways of signal transductions have been identified for the members of TLR family (16,17).

1. Myd88-dependent pathway in TLR2 and TLR4 signaling (Figure 53-3). Myd88 is an adaptor protein that links the IL-1 receptor to a serine-protein kinase (IRAK), critical for IL-1 receptor signal transduction. Studies in macrophages of Myd88^{-/-} mice showed that TLR2- and TLR4-mediated production of proinflammatory cytokines by macrophages (IL-1 β , TNF- α and IL-6) was dependent on Myd88. Detailed genetic and biochemical analyses suggest that IRAK4 and TRAF6 are downstream signaling molecules of Myd88, which is critical for activating TGF- β -activated kinase (TAK-1) and IKK $\alpha\beta$. IKK $\alpha\beta$ then induce IKK $\alpha\beta$ phosphorylation and degradation, which leads to NF- κ B nuclear translocation and activation.
2. TRIF-dependent pathway in TLR3 and TLR4 signaling (Figure 53-3). The ability of poly I:C (TLR3-ligand) and LPS (TLR4-ligand) to induce IFN- β production was found to be independent of Myd88. This leads to the discovery of a second TIR domain-containing adaptor molecule called TIR domain-containing protein inducing IFN- β (TRIF). TLR3 directly transmits signals through TRIF, which interacts with TBK1, RIP1, and TRAF6. TBK1 and IKK $\alpha\beta$ phosphorylate IRF3, which then translocate into the nucleus to activate IFN- β promoters. TLR-4 requires another adapter molecule called TICAM-2 to activate this TRIF-dependent pathway.
3. Myd88-dependent pathway in endosomal TLR7, TLR8, TLR9 signaling. TLR7, TLR8, and TLR9 are located exclusively within the endosomal compartments (Figure 53-3). Recognition of RNA or DNA within the endosomal compartments by TLR7, TLR8, or TLR9, recruit Myd88, which then interact with IRAK4, IRAK1, TRAF6, and IRF7. IRF7 is phosphorylated by IRAK1 and translocates into nucleus to activate the type 1 IFN promoters. TRAF6 also activate three additional pathways including IRF5, MAPKs and NF- κ B, which activate the expression of proinflammatory cytokine genes including IL-1 β , IL-6 and TNF- α .

Dendritic Cells Link Innate and Adaptive Immunity

Dendritic cells are professional antigen-presenting cells, which display an extraordinary capacity to stimulate naïve T-cells and initiate primary immune responses (10,11). This established function of DCs has now offered the hope to apply DC-based immunotherapy for cancers. Recent studies suggest that DCs also play critical roles in the induction of peripheral immunological tolerance, regulate the types of T-cell immune responses and function as effector cells in innate immunity against microbes. In both human and mice, DCs can be grouped into two major subsets: the myeloid conventional DCs (mDCs) and the plasmacytoid DCs (pDCs). Both display functional plasticity depending on the type of activation signals and also their resident microenvironments. However, mDC and pDC display different sets of TLRs and appear to regulate innate and adaptive immunity in different ways.

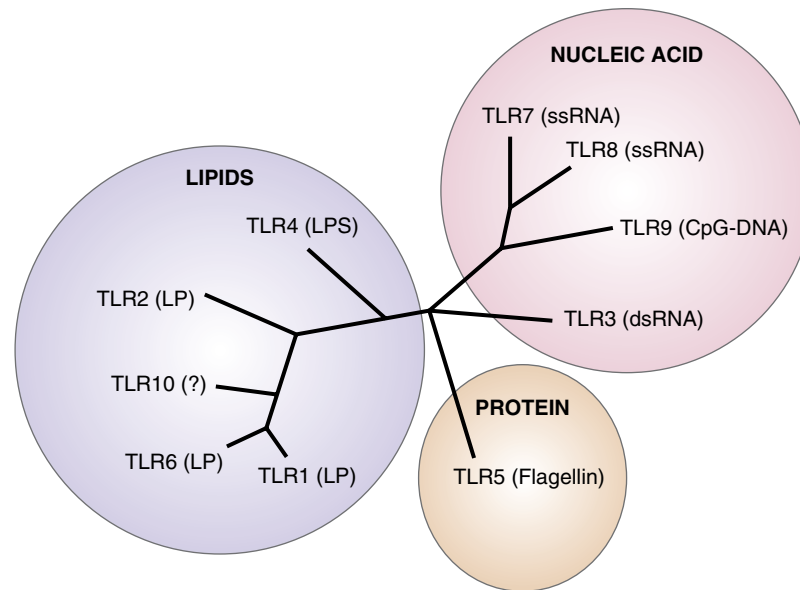


FIGURE 53-1 TOLL-LIKE RECEPTOR (TLR) SUBDIVISION. The phylogenetic analyses of the amino acid sequence and structure of TLRs, suggest that TLRs are evolved to recognize microbial nucleic acid, lipids and proteins.

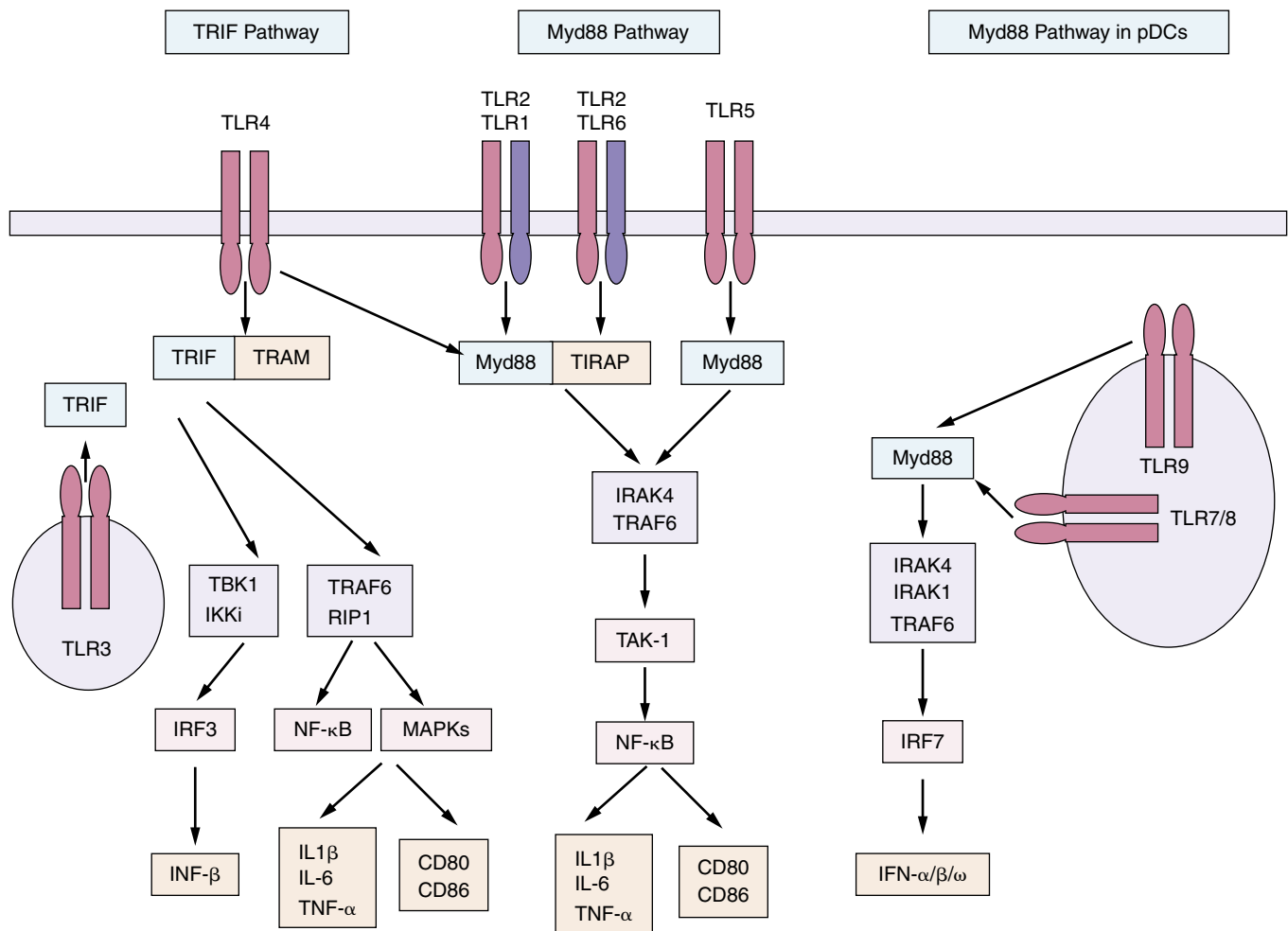


FIGURE 53-2 THREE MAJOR TOLL-LIKE RECEPTOR (TLR) SIGNAL TRANSDUCTION PATHWAYS. (1) Myd88-dependent pathway in TLR2 and TLR4 signaling. (2) TRIF-dependent pathway in TLR3 and TLR4 signaling. (3) Myd88-dependent pathway in endosomal TLR7, TLR8, TLR9 signaling.

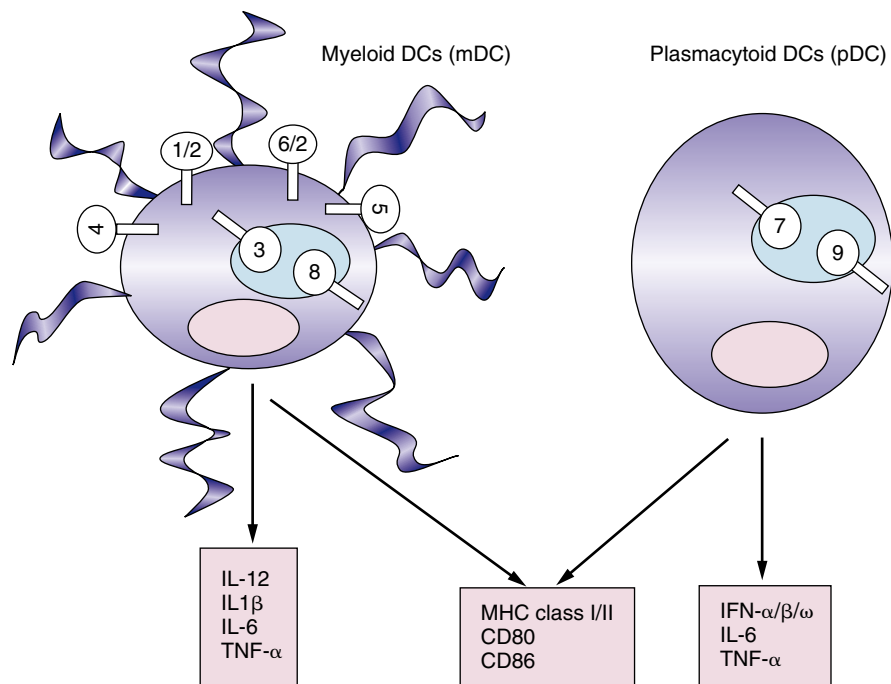


FIGURE 53-3 Toll-like receptor (TLR) expression by human dendritic cells (DCs). Myeloid DCs (mDC) and plasmacytoid DCs (pDC) express different sets of TLRs.

Myeloid DCs and Plasmacytoid DCs Express Different Sets of TLRs

In humans, mDCs can be identified by CD4+CD11c+lineage- and pDCs can be identified by CD4+CD11c-lineage-BDCA-2+ILT7+. Strikingly, while mDCs express TLR2, 3, 4, 5, 6 and 8, pDC only express TLR7 and TLR9. In response to the microbial ligands for the TLR2, 3, 4, 5, 6 and 8, mDCs produce the TH1 polarizing cytokine IL-12 and cytokines IL-1, IL-6, IL-10 and TNF- α , and undergo maturation by upregulation of MHC-class I/class II and costimulatory cytokines CD80, CD83 and CD86 9–11. In response to microbial ligands for the TLR7 and TLR9 and viral infection, pDCs rapidly produce massive amounts of type 1 IFNs, including IFN- α , IFN- β and IFN- ω . Therefore, pDCs are also known as professional type 1 IFN-producing cells (IPCs), which represent the key cell type in antiviral innate immunity. pDCs also have the ability to produce IL-6 and TNF- α upon activation through TLR7 and TLR9, and undergo differentiation into mature DCs that express high MHC class I/class II and costimulatory molecules CD80/CD86 and acquire the ability to prime naïve T-cell activation (9–11).

Functional Plasticity of Dendritic Cells

In humans, DCs were found to display different effector functions in directing T-cell responses that is regulated by the maturation stage of DCs and the maturation signals (10,11). Although

mDCs at mature stage induce T_H1 differentiation and strong cytotoxic T-lymphocyte (CTL) responses, mDCs at immature stage induce IL-10, producing CD4⁺ and CD8⁺ regulatory T cells. Two groups of signals were shown to stimulate immature mDCs to induce Th1 differentiation: (1) LPS derived from gram-negative bacteria (TLR4-L), gram-positive bacteria *Staphylococcus aureus* (SAC) (may trigger multiple TLRs), and double-stranded viral RNA (TLR3-L); and (2) T-cell signals such as CD40L and IFN- γ . Several signals were shown to stimulate immature mDCs to induce Th2 differentiation including epithelial cell-derived cytokine TSLP and helminth *Schistosoma mansoni* egg antigen (18,19). pDC-derived mature DCs also display different effector functions depending on the types of differentiation factors. While pDCs activated by IL-3 and CD40L preferentially promote Th2 differentiation, pDCs activated through TLR7 or TLR9 prime naïve T-cells to produce IFN- γ and IL-10 (11).

Targeting TLRs on DCs to Induce Effective Antitumor Immunity

A major class of adjuvants for vaccines used in humans or in experimental animal models are killed microbials or microbial-derived products that trigger different TLRs (20). The current understanding of TLR biology and DC biology reveals that the immune system has been evolved to fight against microbial pathogens, and does not have an optimal system for sensing

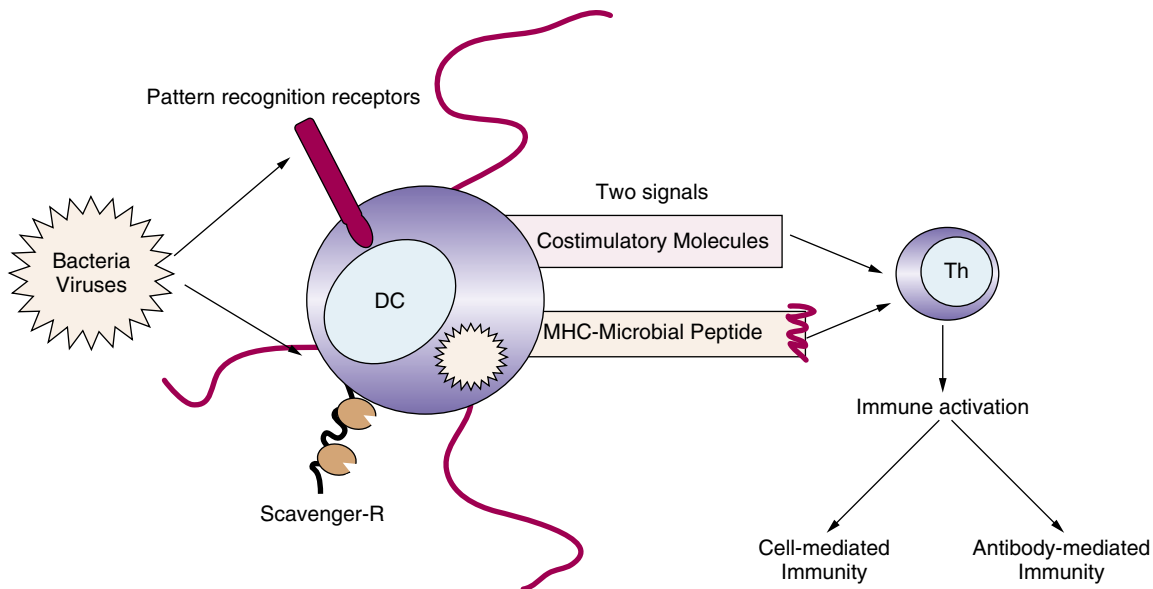


FIGURE 53-4 THE IMMUNE SYSTEM HAS BEEN EVOLVED TO FIGHT AGAINST MICROBIAL PATHOGENS. Dendritic cells (DCs) in the immune system sense microbial infection through pattern recognition receptors, such as the Toll-like receptors (TLRs), which recognized common molecular patterns such as nuclear acid, lipid, proteins, and carbohydrates shared by all microbial organisms. The recognition of microbial molecular patterns by TLRs triggers DCs to up-regulate costimulatory molecules and secrete cytokines, critical for the activation of T-lymphocytes of the immune system. DCs sample microbial antigens by the scavenger receptors through phagocytosis and pinocytosis. The internalized microbial antigens will be processed by proteinases and the resulting short peptides will be presented by the major histocompatibility complex (MHC) class I or class II molecules, allowing recognition by the antigen receptors expressed by T-lymphocytes. T-cells can only be activated when they receive at least two signals from costimulatory molecules and MHC-peptides expressed by DCs to initiate adaptive antimicrobial immune responses.

and effectively responding to cancer (Figures 53-4 and 53-5). Therefore a basic principle (or a dirty trick of immunologists) of developing cancer vaccine is to instruct DCs into thinking that tumor antigens are bacterial or viral antigens by using the microbial adjuvants together with tumor antigens (Figure 53-6). In animal models, stimulation of TLR9 mainly expressed on the

plasmacytoid DCs with CpGs has been shown to increase the immunogenicity of different forms of cancer vaccines, including peptides vaccine, DNA-vaccine, tumor cell-based vaccine and DC-based vaccine (20). Stimulation of TLR4 mainly expressed mDCs by MPL, BCG or murine β -defensin also promote the immunogenicity of cancer vaccines (20). Several studies suggest

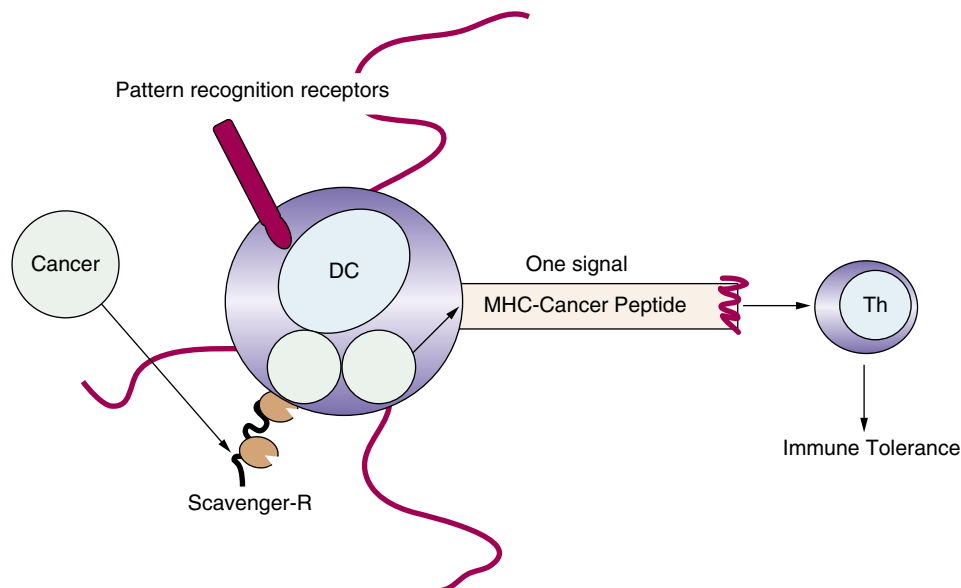


FIGURE 53-5 THE IMMUNE SYSTEM DOES NOT HAVE AN OPTIMAL SYSTEM FOR RECOGNITION OF CANCER. Dendritic cells (DCs) can sample tumor antigens by the scavenger receptors through phagocytosis and pinocytosis. However, cancer cells cannot activate DCs through the pattern recognition receptors. Therefore when tumor antigens are presented by either DCs or by cancer cells to T cells, T-cell activation will not be achieved, because of the lack of the second signal/costimulation.

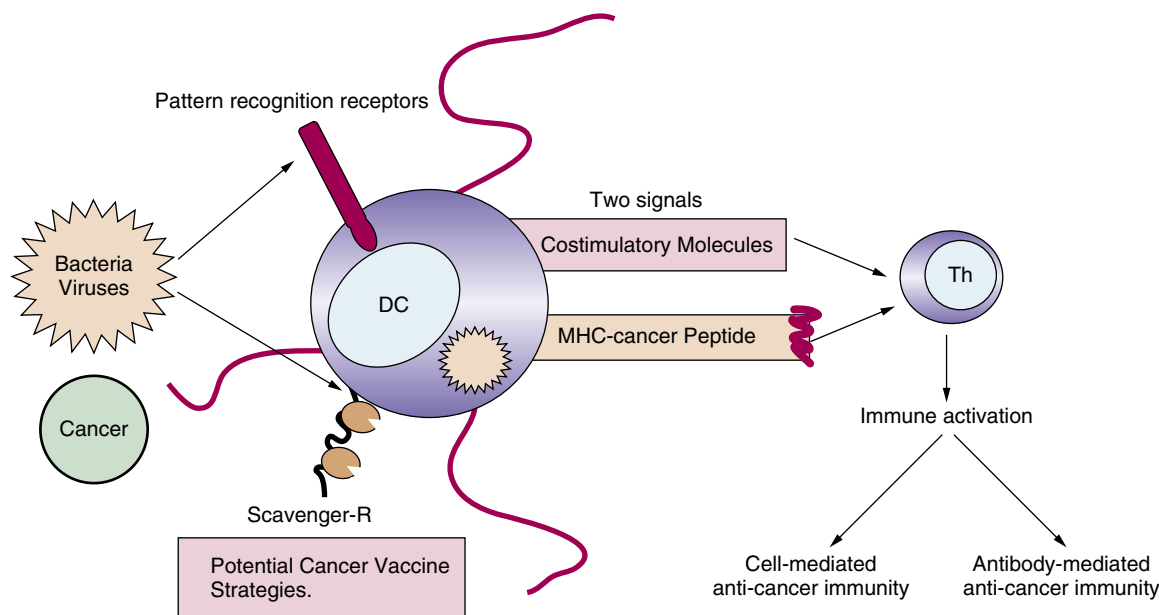


FIGURE 53-6 INSTRUCTING THE IMMUNE SYSTEM TO KILL TUMORS AS IT KILLS BACTERIA AND VIRUSES. The principle of developing cancer vaccine is to combine microbial adjuvants with tumor antigens. Therefore when presenting tumor antigen/peptide in the context of major histocompatibility complex (MHC) to tumor-specific T cells, dendritic cells (DCs) also express the costimulatory molecules and secrete cytokines that will help to activate tumor-specific T-cells, leading to the development of tumor-specific cell-mediated or antibody-mediated adaptive immune responses and immune memory.

that the ability of mDCs to present antigens and activate antigen-specific T-cells can be greatly enhanced by activated pDCs through a type-1 IFN-dependent mechanism in both antiviral immune responses and auto-immune responses (21). We have recently shown that activated pDCs by CpG promote the ability of mDCs to present melanoma antigens to T cells and induce strong tumor-specific CTL responses in vitro and in vivo. In addition, pDCs activated by CpG also strongly activate NK cells, which kills tumor cells and further enhance the ability of mDCs to up take dead tumor cells and cross-present tumor antigens to CD8⁺ T cells (Lu Yanyan et al. Manuscript in preparation).

Immunosuppression Induced by Regulatory T Cells

CD4⁺CD25⁺, naturally occurring regulatory T-cells (T_{reg}) constitute 5% to 10% of peripheral CD4⁺ T cells, which play an essential role in the active suppression of autoimmunity in both humans and rodents. T_{reg} appears to differentiate as a unique T-cell lineage in the thymus from immature T cells expressing T-cell receptor (TCR) with medium to high affinity for self-antigens, which depend on interleukin 2 (IL-2) and costimulatory molecules provided by activated antigen-presenting cells (22–26). Foxp3, a member of the forkhead transcriptional factor family, has been demonstrated to be the master regulator of T_{reg} development in the thymus, as well as T_{reg} suppressive function. Increasing evidence suggest that tumor-specific T_{reg} exist and play an essential role in immune tolerance to tumors, and represent a major hurdle for antitumor immunotherapy. In addition, the tumor microenvironment appears to be the site where tumor-specific infiltrating T-cells are actively converted into tumor-specific T_{reg} (27).

T Regulatory Cells Present in Human Cancers

Immunoregulatory cells and immunosuppressive molecules have been reported in a number of human malignancies, including gastric cancer, Hodgkin disease, and kidney cancer. The following sections detail studies in melanoma and ovarian cancer, highlighting specific mechanisms.

Evidence of Immune Regulation in Melanoma

Melanoma is perhaps the most immunogenic of solid tumors as melanoma-specific CD4⁺ and CD8⁺ T-cells can be isolated directly from tumor deposits. Yet, the tumor metastases grow unabated, thereby implying that immune regulatory mechanisms may be preventing full activation and effector function by tumor-infiltrating T cells. Although it is not clear why melanoma-specific T cells found in tumor deposits are not functioning to eliminate the tumor in vivo, a number of studies have been performed in melanoma patients to help provide some insight into potential regulatory pathways which are active.

Indeed, CD4⁺CD25⁺ T_{reg} have been isolated from melanoma deposits and in fact some Treg lines have been found to be specific for LAGE-1 (28) and ARCT1 (29), expressed by melanoma cells. In a DC melanoma vaccine study, Chakraborty et al. (30) found that vaccine-induced specific CTL responses declined by day 28, and was associated with expansion of CD4⁺CD25⁺IL-10⁺ T-cells. CD4⁺CD25⁺ FoxP3 expressing cells were also found to be overrepresented in melanoma lymph node metastases (31). This may represent a mechanism by which tumors escape the immune system by first generating immunosuppression at the local lymph node site.

Besides $CD4^+CD25^+$ T_{reg} , melanoma cells themselves may produce factors or express receptors which can either induce regulatory immune cells or suppress effector T cells directly. For example, melanoma cells have been found to express IL-10, which is capable of inducing Tr-1 cells, another $CD4^+$ regulatory cell type that can induce T-cell anergy and suppression of immune responses, primarily via the production of high levels of IL-10 and TGF- β (32,33).

PD-L1 (B7-H1) is a member of the B7 family and has been shown to be capable of inhibiting T-cell function and inducing T-cell "exhaustion." PD-L1 was found to be expressed in 22 of 22 melanoma biopsy samples (34). Although PD-L1 expression on renal cancers has been found to correlate with poor prognosis (35), it is as yet not clear whether this association will be found with melanoma.

In addition, other factors produced by tumor cells and immune cells, such as TGF- β , or DO, which depletes tryptophan required by T cells (36,37), may lead to immune suppression.

Ovarian Cancer: Link Between Clinical Outcome, T_{reg} , and Immune Response

A significant body of work has been performed in evaluating the cellular infiltrate of ovarian cancer. Zhang et al. (38) performed immunohistochemical analysis of 186 advanced-stage ovarian cancer patients and found that the 5-year survival was 38% for patients whose tumors were infiltrated by $CD3^+$ T-cells versus 4.5% for patients with an absence of intratumoral T cells. T-cell infiltration was associated with increased interferon- γ , interleukin-2, and specific chemokines within the tumor. However, another group found that not all $CD3^+$ T-cell subpopulations were favorably correlated with outcome in ovarian cancer patients. Accumulation of $CD4^+CD25^+$ T_{reg} cells in ovarian cancer was found to predict decreased survival for stage III and stage IV patients (39). Wolf et al. (40) confirmed and extended these findings by demonstrating that quantitative FoxP3 levels of ovarian biopsies by real-time polymerase chain reaction (PCR) could identify a patient subgroup with decreased overall survival. Finally, Sato and colleagues (41) found that ovarian cancer patients with a higher $CD8^+/T_{reg}$ ratio in tumor tissue had an increased survival. Together, these studies suggest that effector T cells play an important role in mediating an antitumor immune response in ovarian cancer patients while T_{reg} negatively influence this response. This implies that strategies to enhance tumor-specific $CD8^+$ T-cells while decreasing T_{reg} may be effective in improving the outcome for ovarian cancer patients.

Strategies to Block Immunosuppression at the Tumor Site

The elucidation of specific mechanisms of immunosuppression at the tumor site enables the exciting possibility that these pathways may be blocked in order to enhance antitumor immunity.

For example, several strategies can be used to block the suppression of antitumor immunity by T_{reg} :

1. Eliminate T_{reg} by targeting a surface molecule expressed on Treg, such as CD25 (IL-2R α) by a monoclonal antibody or an IL-2-toxin fusion protein (Ontak).
2. Eliminate T_{reg} by lymphocyte depletion with cytotoxic drugs.

3. Block the mediators of suppression by T_{reg} , such as IL-10, TGF- β , and CTLA-4 (Table 53-1).
4. Activate DCs to express IL-6 that will block the function of T_{reg} , or to express GITRL that will activate tumor-specific T cells to become resistant to T_{reg} -mediated immunosuppression.
5. Target TLR8 expressed on T_{reg} , which will block the inhibitory function of T_{reg} .

Further progress will require a deeper understanding of the regulatory immune cell subpopulations and the ability to determine the most relevant suppressive pathways in individual patients, since there are numerous potential mechanisms of immune regulation. As this knowledge base increases, specific interventions to block immune regulation can be developed.

Autoimmunity and Antitumor Immunity

T-Cells Can Recognize Self Antigens Expressed By Tumors

Over the past 10 years, numerous tumor antigens have been described that can be recognized by T cells (42). These have been identified by two major methods: (1) molecular cloning using tumor antigen-specific T cells derived from cancer patients and (2) analysis of candidate antigens based on gene expression and molecular profiling of tumors. Many of these antigens are expressed on normal tissues and are therefore considered to be "self" antigens. Examples of this class of antigen include melanocyte differentiation antigens, which are expressed on melanoma cells as well as normal melanocytes. These include tyrosinase, MART-1, gp100, and TRP-1 (42). Differentiation antigens, expressed on tumor as well as the normal tissue of origin, can be targeted in immunotherapeutic strategies, as long as the normal tissue is nonessential. Mutated antigens, endogenous retroviral antigens, and antigens expressed in tumor and testis have also been described to be expressed in the context of MHC class I and class II molecules, capable of being recognized by $CD8^+$ and $CD4^+$ T-cells, respectively. Although mutated antigens may be more easily recognized than "self" antigens, it is also possible that peripheral tolerance develops against the mutations, since costimulation may be absent in tumors, which are often present for many years prior to clinical diagnosis.

Autoimmunity and Response to Immunotherapy

Because many tumor antigens are also expressed by normal host tissues, such as normal melanocytes in the case of melanoma antigens, obtaining an effective antitumor immune response requires overcoming self tolerance. Indeed, in some settings, effective immunotherapy has been correlated with autoimmune responses against host tissues.

Relationship Between Vitiligo and Response to IL-2 in Melanoma Patients

Interleukin-2 (IL-2), a cytokine that can stimulate the proliferation of T cells, can result in significant long-lasting regression

Table 53-1 Potentially Targetable Immunoregulatory Molecules

	Molecule	Cellular Expression	Mechanism of Action
Membrane bound	CTLA-4	Helper T Cytotoxic T	Provides co-inhibitory signaling during naïve T-cell priming
		T-reg	Induces local typtophan metabolism by DCs, directly inhibits T-cells
	PD-1	Helper T Cytotoxic T	Inhibits T-cell proliferation, cytokine production and cytotoxicity
Soluble	IL-10	Tumor Tr1	Regulates growth and differentiation of a wide variety of immune cells
	IL-13	iNKT	Induces immature myeloid cells to produce TGF- β
	TGF- β	Tumor Tr1, T _{reg} Immature myeloid	Directly suppresses proliferation of antigen-activated T-cells
	VEGF	Tumor	Blocks DC differentiation and maturation, leading to accumulation of iDC and iMC
	IDO	Tumor Dendritic	Depletes local typtophan, inhibiting T-cell proliferation
	ARG1	Tumor Immature myeloid	Depletes local arginine, inhibiting CD3 ζ expression and T-cell activation
	iNOS	Tumor Immature myeloid	Generates nitric oxide, inhibiting T-cell priming, proliferation, and cytotoxicity

of disease in some patients with metastatic melanoma and renal cell cancer (see following sections). Interestingly, Rosenberg (43) found that while none of 104 renal cancer patients treated with high-dose IL-2 developed vitiligo, the immune destruction of normal melanocytes, 11 of 74 melanoma patients treated with high-dose IL-2 developed vitiligo (43). Vitiligo was seen in 26% of melanoma patients who demonstrated objective tumor response to IL-2, whereas no vitiligo was seen in patients who did not respond to the IL-2. This suggests that tolerance to self-antigens can be overcome in the induction of an effective antitumor immune response.

Correlation Between Autoimmunity and Clinical Response to Anti-CTLA-4 Antibody

Another approach that has been shown to be effective in inducing regression of metastatic disease in melanoma patients is the use of an antibody that specifically blocks the CTLA-4 molecule, which is rapidly up-regulated on effector T cells following activation. CTLA-4 suppresses immune responses by binding to the costimulatory molecule B7, thereby preventing optimal T-cell activation, which requires interactions between CD28 and B7. Interestingly, clinical response to anti-CTLA-4 antibody has been found to be correlated with the development of autoimmune side effects, which include colitis, dermatitis, uveitis, enterocolitis, hepatitis, and hypophysitis, which is an inflammation of the pituitary gland (44). The clinical response rate in patients who demonstrated significant autoimmunity (grade III/IV) was 36% (five of 14), while it was only 5% (two of 42) in the group of patients who did not demonstrate significant autoimmunity. Again, this suggests that immune interventions that allow the breaking of tolerance to self antigens

can also result in tumor destruction through immune recognition of self proteins.

Perhaps future strategies combining anti-CTLA-4 with an effective vaccine may skew the immune response towards tumor and away from normal tissues, thereby enabling a more specific antitumor reaction.

Cancer Vaccines, Cytokines, and Immunotherapy

Cytokine Therapy of Cancer

Perhaps the strongest evidence that immune responses can result in a significant antitumor effect in patients is the fact that a subset of patients with metastatic melanoma can have complete tumor regressions and long-term survival following the administration of the T-cell growth factor interleukin-2. Of 270 patients with metastatic melanoma treated with high-dose IL-2, 43 patients (16%) had an objective response (complete or partial response; 45). More importantly, 60% of those patients who achieved a complete response had prolonged disease-free intervals and long-term survival (>10 years). This demonstrates that it is possible, by activation of the immune system, to induce clinically meaningful responses in patients. Future studies are needed to focus on understanding more fully the mechanism of response in patients so that improved strategies can be designed which will result in higher response rates and improved survival in patients.

Another cytokine with significant clinical activity is interferon α , a type I interferon which is normally produced by plasmacytoid

dendritic cells following viral infections. In randomized trials, high-dose interferon therapy has been shown to decrease tumor recurrence and increase survival in stage III melanoma patients (following surgical resection of tumor-positive lymph nodes; 46). A recent study investigated prognostic markers in melanoma patients receiving interferon α in the adjuvant setting (47). Patients who, during interferon therapy, developed autoantibodies, including antithyroid, antinuclear, or anticardiolipin antibodies, had significantly enhanced survival compared to patients who did not develop signs of autoimmunity. This again highlights the link between immunotherapy and autoimmunity as discussed previously. In addition, it suggests that, although interferon has pleiotropic effects on tumor and host tissues, such as effects on tumor vasculature and direct inhibitory effects on tumor proliferation, the mechanism of action in melanoma patients is by stimulating anti-tumor immunity by breaking tolerance to self antigens. This may be due to the effects of type I interferons on antigen-presenting cells or T cells. In addition, interferon α can up-regulate MHC molecules on tumor cells, thereby rendering them better targets for T-cells.

Current Status of Cancer Vaccines

With clear evidence that the immune system can play an important role in mediating a clinical response in cancer patients, current efforts are focused on developing cancer vaccines to enhance efficacy as well as specificity, since nonspecific immune stimulation as discussed can result in autoimmunity. Two major approaches have been pursued in cancer vaccine strategies, utilization of whole tumor cells or the use of specific tumor antigens (Table 53-2).

Whole-Cell Cancer Vaccine Strategies

Cancer vaccine strategies have been performed using derivatives of both autologous and allogeneic tumor cells. Although the use of autologous tumor cells is more labor intensive in that vaccines need to be prepared individually for each patient, autologous tumor has the advantage of containing specific mutations for that patient, which may be seen as more foreign compared with shared, self-antigens. Cell lysates fed to autologous dendritic cells, isolation of heat shock proteins bound to autologous antigens, and gene modification of autologous tumor with immune-enhancing cytokines have been evaluated in clinical trials. In murine models, transduction of tumor cells to express GM-CSF results in enhanced anti-tumor immune responses against parental nontransduced tumor cells (48). Antitumor activity of GM-CSF-expressing tumors was found to be dependent on host bone-marrow-derived antigen-presenting cells (49), CD1d-restricted NKT cells, CD4⁺ and CD8⁺ T-cells, and antibodies (50).

Antigen-Specific Vaccine Approaches

As discussed previously, numerous tumor antigens have now been identified that can be recognized by T-cells in the context of MHC class I and class II molecules. Many of these antigens

are shared among specific types of tumors, and represent a feasible target for vaccine development. Antigen-specific vaccine approaches have included the use of specific peptide epitopes, whole proteins, and recombinant DNA and viral vaccines. The advantage of whole-protein and recombinant approaches using the entire antigen gene is that multiple class I and class II epitopes may be presented (51). However, whole proteins have been expensive and challenging to produce clinically, and viral vaccines can induce neutralizing antibodies that prevent the efficacy of serial doses of vaccine. Peptide vaccines, in combination with specific adjuvants, have demonstrated potential clinical efficacy in the case of CML (52), and have been the most consistent method of inducing high levels of circulating antigen-specific T cells (53). However, in the case of patients with metastatic melanoma, high levels of tumor-specific T cells in the blood do not always result in tumor regression (54). Therefore, future efforts are focused on stimulating stronger and more effective T-cell priming through the use of specific adjuvants such as TLR agonists, using concepts learned from basic studies of antiviral immunity as described above. This may result in T cells with increased affinity and specificity as well as enhanced memory and effector function. One method to more carefully manipulate the specific phenotype of tumor reactive immune cells is to generate and select specific T-cells in the laboratory followed by reinfusion into patients. This is termed adoptive immunotherapy.

Adoptive Immunotherapy of Cancer

One of the most significant recent advances in clinic immunotherapy has been the adoptive transfer of tumor-reactive lymphocytes. A number of lines of evidence have demonstrated the clinical effectiveness of this approach, including donor-lymphocyte infusion following allogeneic bone marrow transplantation (55), treatment of metastatic EBV-driven lymphoproliferative tumors (56), and therapy of metastatic melanoma.

In the setting of metastatic melanoma, T cells can be found at the tumor site (tumor-infiltrating lymphocytes, or TILs) that are specific for melanoma antigens, such as MART-1 and gp100 (Figure 53-7). However, because the tumor is growing, these T cells are nonfunctional or the tumor is resistant to recognition or lysis. However, when TIL are expanded *ex vivo* and reinfused, clinical regressions are seen in patients with metastatic disease. A number of reasons may explain the effectiveness of *ex vivo* expanded lymphocytes compared to endogenous T-cells. First, large numbers can be generated in the laboratory, which may be difficult to achieve *in vivo*. Second, expanding the lymphocytes *ex vivo* takes them out of the suppressive tumor microenvironment. Finally, growth *ex vivo* may allow reactivation of lymphocytes rendered anergic or nonfunctional due to *in vivo* toleragenic mechanisms. Initially, transfer of tumor-reactive T cells alone resulted in response rates of greater than 20%. *In vitro* predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2 (57), but clinical regressions were often transient in nature and it was clear from gene marking studies that the T cells did not survive long *in vivo* (58).

Table 53-2 Advantages and Disadvantages of Various Cancer Vaccine Approaches

	Whole-Tumor Vaccines			Antigen-Specific Vaccines			
		Autologous	Allogeneic	Peptide	Whole Protein	Dna	Recombinant viral
Advantages	Easily produced		x	x		x	
	Contains shared antigens, so can be used on multiple patients		x	x	x	x	x
	Contains multiple antigens, including unknown antigens	x	x				
	Contains multiple epitopes from the same antigen	x	x		x	x	x
	Contains individual mutated antigens	x					
Disadvantages	Costly	x			x		x
	Needs to be produced for individual patients	x					
	Stimulates a neutralizing antibody						x
	Low levels of antigen expression	x	x			x	
	Potentially stimulates competing anti-viral immune responses						x

More recently it was found that transient lymphodepletion using cytoxan and fludarabine prior to T-cell infusion resulted in improved response rates and T-cell survival. Eighteen (51%) of 35 patients with metastatic melanoma exhibited objective responses following lymphodepletion and adoptive T-cell transfer. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes (59). Adiotuve cell transfer therapy following nonmyeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma (60). Substantial numbers of infused lymphocytes were found in the circulation in some patients more than 2 years after infusion. This dramatic improvement following lymphodepletion may be due to a number of potential mechanisms including elimination of regulatory T cells and enhancement of lymphocyte homeostatic proliferation.

Current studies of adoptive immunotherapy are focused on optimizing the generation of T cells ex vivo and their proliferation in vivo. For example, murine models suggest that proliferation of adoptively transferred T cells can be greatly enhanced in vivo by addition of active immunization, such as dendritic cell vaccines

(61). In addition, significant efforts are focused on the introduction of novel genes into T cells to enhance their ability to recognize, migrate to, and eliminate tumor cells. In a recent study, nonspecific peripheral blood T cells were gene modified with a melanoma-specific T-cell receptor and then reinfused into melanoma patients. Two patients (of 17) demonstrated objective clinical responses, demonstrating that gene modification of lymphocytes is a feasible and potentially efficacious maneuver, although future studies are focused on enhancing the overall response rate (62).

Because tumor-reactive lymphocytes are naturally found in melanomas, much of the work in adoptive therapy has focused on this disease. Although tumor-reactive lymphocytes are rarely found in other common cancers, antibodies that recognize these tumors in a relatively specific fashion have been described. Therefore, chimeric receptors have been designed using antibody variable regions extracellularly fused to T-cell signaling chains intracellularly. The initial studies demonstrated the ability to redirect T-cell specificity in vitro against ovarian cancer (63). Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the

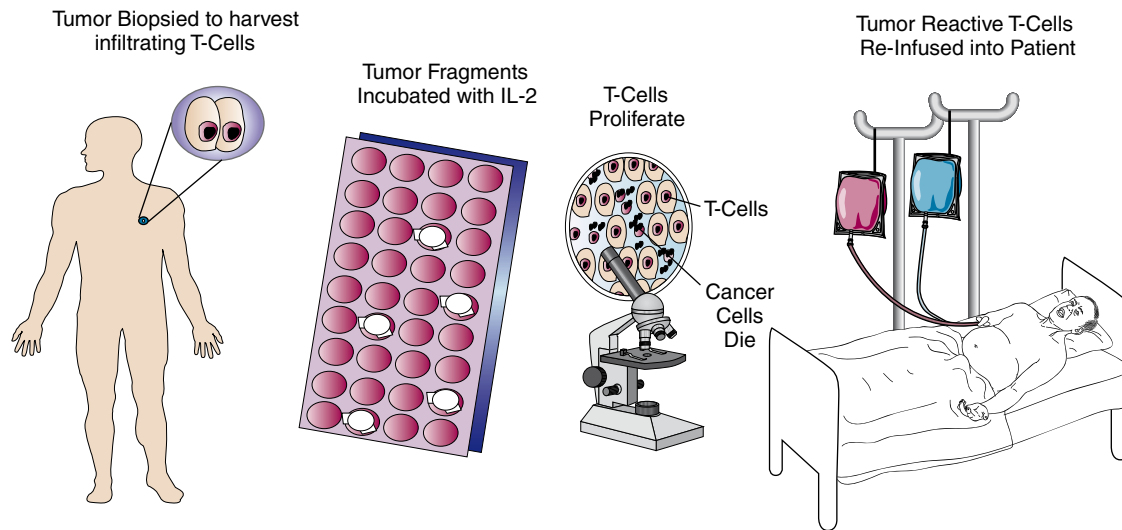


FIGURE 53-7 EXPANSION OF TUMOR INFILTRATING LYMPHOCYTES (TILs) FROM MELANOMA. T cells capable of specifically recognizing tumors can be found in some metastatic melanomas. When tumor fragments are cultured in the T-cell growth factor interleukin-2 (IL-2), the T cells expand and destroy the tumor cells in vitro. The expanded TIL can then be reinfused into patients with metastatic melanoma, in combination with interleukin-2.

Fc receptor gamma chain, but subsequent receptors have been designed that recognize HIV (64) and a number of other tumor types (65,66).

Besides redirecting T-cell recognition, the introduction of novel genes into effector lymphocytes may be utilized to enhance other functional properties, such as T-cell migration to the site of tumor (67) and activation status (Figure 53-8). For example, Kershaw et al. (6) introduced the chemokine receptor gene *CXCR2* into T-cells and demonstrated the ability of these modified cells to migrate toward chemokines

produced by tumor cells. In addition receptor genes have been introduced with signaling chains containing costimulatory sequences to enhance T-cell activation. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCR-zeta/CD28 receptor (68).

In summary, the infusion of tumor-reactive, ex vivo-expanded T cells has clearly demonstrated the effectiveness of T-cell-mediated immunity in the treatment of patients with metastatic cancer. Future studies will focus on enhancing response rates through generation of T cells with greater activity and ability to

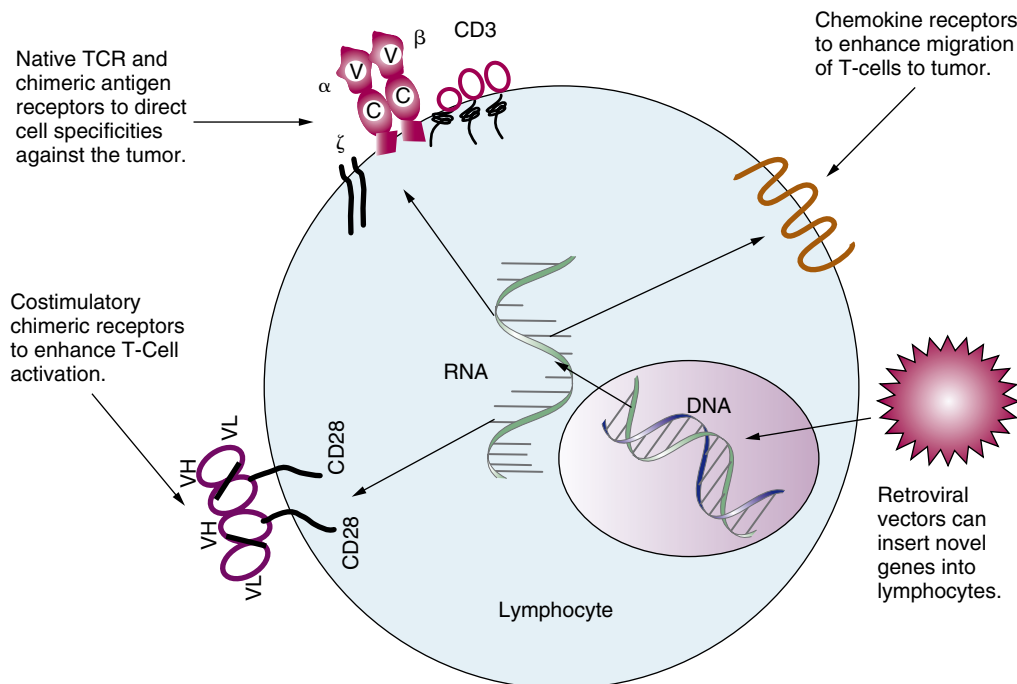


FIGURE 53-8 INSERTION OF GENES INTO LYMPHOCYTES TO ENHANCE ANTITUMOR PROPERTIES. Genes can be inserted into T cells using retroviral vectors. These genes can endow the T cells with novel properties. Using genes encoding native T-cell receptor or chimeric antibody/T-cell receptor genes, T-cells can gain the ability to recognize new targets. Using costimulatory receptor genes, T-cell activation can be enhanced. Finally, using chemokine receptor genes, T cells can more efficiently migrate to tumor sites.

migrate to tumor, durability of response by improved maintenance of T cells in vivo, and the use of T cells in nonmelanoma tumors by redirecting cells with native T-cell receptor or novel chimeric receptor genes.

Future Cancer Immunotherapies Will Utilize Basic Principles of Cellular Immunity

Although the adoptive transfer of carefully selected T-cell populations or the use of optimized vaccine strategies may allow for the presence of more potent T cells in vivo, this must be coupled with enhanced conditioning of the tumor microenvironment to allow migration and function of specific T cells. In successful immune responses against viruses, a proinflammatory state exists in the infected site by TLR activation through specific viral

components as previously discussed. This inflammation induces the up-regulation of specific adhesion molecules on endothelial cells that will in turn mediate trafficking of primed T cells into the infected site. In addition, the proinflammatory state may enhance effector T-cell function while limiting the effects of immune regulation. Successful immunotherapy will require enhanced understanding of the interplay between immune cell subpopulations that results in optimal T-cell induction as well as tumor site conditioning that will allow enhanced efficacy of the T cells in the tumor microenvironment. This multidimensional approach will require the rationale combination of new and existing clinical agents.

Finally, although many of the initial principles have been developed using immunogenic tumors, such as melanoma, future goals include the application of these principles to develop immunotherapeutic approaches against common cancers such as breast and colon cancer, which will be possible as additional antigens capable of being recognized by T cells are isolated from these tumor types.

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Cancer-Specific Vaccines

Since the original reports of Jenner over two centuries ago, prophylactic vaccination against infectious diseases has been one of the most influential medical interventions. Cancer vaccination, an immunotherapy approach applied to patients with established cancer, has tremendous potential based on the ability of T cells and antibodies to specifically recognize cancer antigens and kill cancer cells expressing these antigens (Figure 54-1). However, at the time of this writing, human cancer vaccines have failed to yield U.S. Food and Drug Administration (FDA) approval despite multiple phase 3 clinical trials over the past two decades. Despite the clinical failures of cancer vaccines to date, continuing molecular definition of tumor-specific and tumor-selective antigens, new vaccine platforms that selectively target and activate dendritic cells and preclinical results with combinations of vaccination together with other immune modulators have generated renewed optimism that cancer vaccination will ultimately take its place among the pantheon of cancer therapies.

Prophylactic vaccines for infectious diseases have two major features distinct from cancer vaccines that have been responsible for their success. First and foremost, because the infectious agent being vaccinated against is known, prophylactic vaccines given before exposure to the infectious agent target a naïve immune system. In contrast, multiple studies over the past decade have demonstrated that the major agent of adaptive immunity to tumors—T cells—have been rendered tolerant to tumor antigens in patients with established cancer. Therefore, to be successful, therapeutic cancer vaccines must break tolerance to generate therapeutically meaningful immune responses. Secondly, virtually all prophylactic vaccines work through the generation of neutralizing antibodies that prevent the initial stages of infection or neutralize toxins produced by infectious microbes. Thus, developers of prophylactic vaccines can safely use the surrogate endpoint of neutralizing antibody responses to determine whether their candidate vaccine will have a high likelihood of successfully preventing the pathologic consequences of infection. In contrast, there is little evidence that antibody responses generated by cancer vaccines play a significant role in antitumor responses, with the exception of blocking antibodies generated against certain “oncogenic” receptor tyrosine kinases (such as EGFR and HER2/Neu) known to be important in the growth and survival of many tumors. Instead, proper activation of high-affinity tumor-specific T cells appears to be a critical outcome for therapeutic cancer vaccines to display clinical efficacy.

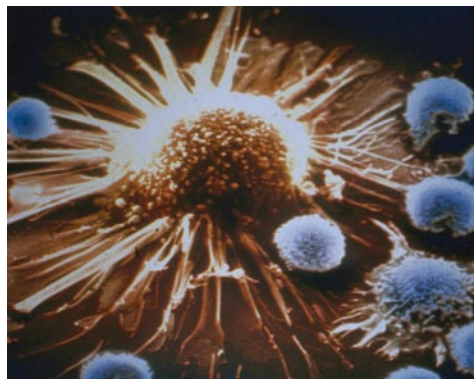
Adoptive T-cell transfer trials using ex vivo-expanded tumor-specific T cells have demonstrated clear proof of principle that activated tumor-specific T cells can induce tumor regressions, even in patients with bulky metastatic cancer (1,2). Because adoptive T-cell transfer is prohibitively expensive, labor intensive, and extremely difficult to standardize, it is an immunotherapy approach unlikely to be broadly exportable. Most cancer immunotherapy efforts, including those that involve vaccination, seek to activate and expand tumor-specific T cells in vivo via various manipulations involving standardized reagents. The major barriers to be overcome are induction of tolerance among tumor-specific T cells and a tumor microenvironment that has developed to resist infiltration and attack by activated tumor-specific T cells. Although these two barriers represent significant hurdles to successful cancer immunotherapy, the elucidation of specific molecular mechanisms for tolerance induction as well as immune inhibition within the tumor microenvironment have led to the generation of specific combinatorial approaches to cancer therapy (3).

Diversity

T cells (TCR) - 10^{18}
Antibodies - 10^{22}

Specificity

Can distinguish a
single methyl group



Weaponry

NO, superoxides,
HOCl, H₂O₂,
FasL, TRAIL,
Perforin,
Granzyme B,
Myeloperoxidase
complement
phagocytes

FIGURE 54-1 THE IMMUNE SYSTEM AS A POTENTIALLY POTENT WEAPON AGAINST CANCER. Depicted is a scanning electron micrograph of a tumor-specific cytotoxic T cell attaching to a tumor cell and injecting enzymes that activate the apoptotic cascade. Based on the high degree of diversity of T-cell receptors (TCRs; T cells) and antibodies (B cells), as well as the diverse armamentarium of cytotoxic molecules the immune system possesses great capacity as an anticancer weapon. Specifically activating and targeting the immune system against tumors has been a longstanding challenge.

Tumor Antigens

The most important concept underpinning efforts to develop therapeutic vaccines for cancer is the antigen difference between tumors and normal cells, offering the capacity for specific recognition by T cells (Table 54-1). It is now established that tumors differ fundamentally from their normal cell counterparts in both antigenic composition and biologic behavior. Genetic instability, a basic hallmark of cancer, is a primary generator of tumor-specific antigens. The most common genetic alteration in cancer is mutation, arising from defects in DNA damage repair systems of the tumor cell (4–10). Recent estimates from genome-wide sequencing efforts suggest that every tumor contains a few hundred mutations in coding regions (11). Additionally, deletions, amplifications, and chromosomal rearrangements can result in new genetic sequences resulting from juxtaposition of coding sequences not normally contiguous in untransformed cells. The vast majority of these mutations occur in intracellular proteins and thus, the “neoantigens” they encode would not be readily targeted by antibodies. However, the major histocompatibility complex (MHC) presentation system for T-cell recognition makes peptides derived from all cellular proteins

available on the cell surface as peptide MHC complexes capable of being recognized by T cells. Based on analysis of sequence motifs, it is estimated that roughly one third of the mutations identified from genome sequencing of 22 breast and colon cancers (11) were capable of binding to common HLA alleles based on analysis of sequence motifs (J.P. Allison, personal communication, July 2007).

Most tumor-specific antigens derived from mutation as a consequence of genetic instability are unique to individual tumors. The consequence of this fact is that antigen-specific immunotherapies targeted at most truly tumor-specific antigens would by necessity be patient specific. However, there are a growing number of examples of tumor-specific mutations that are shared. The three best studied examples are the Kras codon 12 G→A (found in approximately 40% of colon cancers and more than 75% of pancreas cancers), the BrafV599E (found in roughly 70% of melanomas), and the P53 codon 249 G→ mutation (found in ≈50% of hepatocellular carcinomas; 12–15). As with nonshared mutations, these common tumor-specific mutations all occur in intracellular proteins and therefore require T-cell recognition of MHC-presented peptides for immune recognition. Indeed, both the Kras codon 12 G→A and the BrafV599E mutations result

Table 54-1 Different Categories of Human Tumor Antigens

Category of Tumor Antigen	Advantages as Vaccine Antigen	Disadvantages as Vaccine Antigen
Arising from common Oncogene/Tumor Suppressor Gene Mutation Examples: Kras G12A (colon, pancreatic) BrafV599E (melanoma) P53 G249T (hepatoma)	Highly tumor specific Antigen specific T cell repertoire likely to be present Necessary for tumor growth/maintenance and therefore cannot be eliminated by tumor as an escape mechanism	Highly limited epitopes (encompassing mutation site) available for HLA presentation in a given patient Limited number of examples of common mutations identified (but may increase with tumor genome sequencing efforts)
Cancer/Testes Antigens Examples: MAGE 1–12 (many tumors) NY-ESO-1 (many tumors) RAGE (renal, SSCHN, leukemia, others) GAGE (HNSCC, others)	Highly tumor specific (only normal tissue expression is in testes) Many T cell epitopes Shared among many different tumor types Tolerance to antigens may be limited	Unnecessary for tumor growth/maintenance and therefore easily lost as an escape mechanism Heterogeneous expression within tumors, many tumor cells negative
Upregulated in cancers via epigenetic mechanisms Examples: CEA (gastrointestinal cancers) WT-1 (Wilms' tumor, leukemias, lymphomas) Mesothelin (pancreatic, ovarian, mesothelioma) HER2/Neu (Breast, ovarian cancer)	Shared among many tumors May be necessary for tumor growth/maintenance and therefore cannot be eliminated by tumor as an escape mechanism Some cell membrane antigens in this category may be additionally targets for monoclonal antibodies	Not tumor specific. Collateral damage to normal tissues may result from strong induced immune response Because they are self antigens, immune tolerance may blunt vaccine induced immune responses
Tissue specific antigens shared by tumor Examples: Tyrosinase (melanoma) MART1/MelanA (melanoma) gp100 (melanoma) PSA (prostate) PAP (prostate)	Shared by many tumors Collateral damage to normal tissue counterparts may be highly acceptable for tumors derived from dispensable tissues (i.e., melanocytes, prostate)	Because they are self antigens, immune tolerance may blunt vaccine induced immune responses
Viral antigens expressed in tumors (or precancer) Examples: HPV E6,E7 (cervical cancer) EBV EBNA-1, LMP1,2 (Hodgkins, nasopharyngeal cancer)	Highly tumor specific Large number of potential epitopes Antigen-specific T cell repertoire likely present Necessary for tumor growth/maintenance	Limited to tumors caused by viruses

in neopeptides capable of being recognized by HLA class I- and class II-restricted T cells (16–20).

The other major difference between tumor cells and their normal counterparts derives from epigenetics (21). Global alterations in DNA methylation as well as chromatin structure in tumor cells results in dramatic shifts in gene expression. All tumors overexpress hundreds of genes relative to their normal counterparts, and in many cases, turn on genes that are normally completely silent in their normal cellular counterparts. Overexpressed genes in tumor cells represent the most commonly targeted tumor antigens by both antibodies and cellular immunotherapies. This is because, in contrast to most antigens derived from mutation, overexpressed genes are shared among many tumors of a given tissue origin or sometimes multiple tumor types. For example, mesothelin, which is targeted by T cells from vaccinated pancreatic cancer patients (22), is highly expressed in virtually all pancreatic cancers, mesotheliomas, and most ovarian cancers (23,24). Although mesothelin is expressed at low to moderate levels the pleural mesothelium, it is not expressed at all in normal pancreatic or ovarian ductal epithelial cells.

The most dramatic examples of tumor-selective expression of epigenetically altered gene are the so-called cancer-testis antigens (25). These genes appear to be highly restricted in their expression in the adult. Many are expressed selectively in the testis of males and are not expressed at all in females. Expression in the testis appears to be restricted to germ cells and in fact, some of these genes appear to encode proteins associated with meiosis (26–28). Cancer-testis antigens therefore represent examples of widely shared tumor selective antigens whose expression is highly restricted to tumors. The tumor distribution of cancer-testes antigens is highly diverse, with expression of a given antigen at various levels in many different tumor types. The most commonly explored antigens in human vaccine trials are Mage3 and NY-ESO-1, all of which demonstrate a broad distribution of tumor expression in solid and liquid tumors. Many cancer-testis antigens are recognized by T cells from nonvaccinated and vaccinated cancer patients (25). From the standpoint of immunotherapeutic targeting, a major drawback of the cancer-testis antigens is that none appear to be necessary for tumors' growth or survival. Therefore, their expression appears to be purely the consequence of epigenetic instability rather than selection and antigen-negative variants are easily selected out in the face of immunotherapeutic targeting.

Tissue-specific antigens expressed by tumors represent another category of shared "tumor antigen" and have been popular targets of cancer vaccination. A number of important cancers such as melanoma and prostate cancer continue to express antigens selective to their tissue of origin. Thus, melanomas express many of the melanocyte-specific enzymes involved in melanin biosynthesis (such as tyrosinase, gp100, TRP-2, etc.; 29–32). Prostate cancers express prostate-specific antigens such as prostatic acid phosphatase and prostate-specific antigen. Because these tissues are dispensable to life, the development of a strong T-cell response against these antigens that resulted in tumor elimination associated with autoimmune responses against the normal tissue counterparts would be a therapeutically acceptable tradeoff. Although there has been

limited credible demonstration of robust T-cell responses against prostate antigens, the most common T-cell responses identified from melanoma patients are indeed specific for melanocyte-differentiation antigens, many of which are part of the specialized melanin-producing lysosome of the melanocyte termed the "melanosome" (25,29–32). The correlation between tumor response and induction of vitiligo (autoimmune destruction of melanocytes) in many immunotherapy trials indeed suggests the notion that induction of enhanced immune responses against tissue-specific antigens is therapeutically plausible in the setting of tumors of dispensable tissues (33).

A final important category of tumor antigen encompasses viral antigens for virus-associated cancers. In some ways, these represent the best example of shared tumor antigens that are "non-self" antigens. It is now well recognized that cervical cancer is associated with infection and integration of human papillomavirus (HPV)–16, –18 and other strains, and expression of 2 HPV antigens: E6 and E7 (34,35). A number of B-cell lymphomas and solid tumors are associated with Epstein Barr virus (EBV) infection and integration with expression of certain EBV antigens. Examples of EBV-associated tumors in immunocompetent individuals include nasopharyngeal carcinoma and Hodgkin disease (36–38). EBV is the major cause of lymphomas in immunodeficient patients. Adoptive T-cell transfer experiments in immunodeficient patients with EBV-associated T-cell lymphomas demonstrate the capacity of these T cells to induce clinically significant antitumor responses. In addition to cancer, a number of chronic viral infections leading to premalignant conditions such as hepatitis B virus (HBV) and hepatitis C virus (HCV) involve the expression of viral antigens that could be potentially targeted by vaccines as a cancer prevention approach (39–41).

Principles of Cancer Vaccine Design

The immune system of patients with cancer is constantly exposed to antigens expressed by their tumors. The fact that the tumor is growing progressively implies that the capacity of the natural immune response to eliminate the tumor has been subverted. A large body of work, whose description is beyond the scope of this chapter, indicates that tumors indeed use many mechanisms to evade and escape immune recognition. Mechanisms of immune escape and evasion involve ongoing mutation of antigens, expression of molecules that inhibit immune effector function within the tumor microenvironment, loss of molecules involved in presentation of antigens to T cells (i.e., MHC molecules), loss of responsiveness to the interferon system, and most importantly, tolerance induction. Induction of tolerance among tumor-specific T cells is an active process mediated by specific oncogenic pathways (such as STAT3) and may be the single most important barrier to successful therapeutic cancer vaccination (42,43). Thus, cancer vaccines must do more than induce immunity among the naïve T-cell repertoire (the goal of prophylactic vaccines); they must break tolerance and induce effector responses that can succeed within the inhibitory microenvironment of the tumor.

Given the fundamental tenet that cancer vaccines work via the induction of tumor-specific T cell responses, there are two critical cell types whose biology is central to the efficacy of a given vaccine. One, obviously, is the T cell. The other is the antigen-presenting cell (APC). It is the APC that is primarily targeted by the vaccine since this is the cell that processes and presents peptide antigens to T cells while providing costimulatory signals critical for T-cell activation.

Growth and Differentiation Programs of Dendritic Cells

The central theme among cancer vaccination strategies is enhancement of modulation of APC function. This is based on the concept that the quantitative and qualitative characteristics of T-cell responses to antigen depend on the signals they receive from the APC. Among the major bone marrow–derived APC subtypes (B cells, macrophages, and dendritic cells [DCs]), the DC has emerged as the most potent APC type responsible for initiating immune responses (44,45).

As virtually all phases of DC differentiation and function can be modulated by engineered vaccines, it is important to understand the molecular signals that regulate their role in activation of T-cell–dependent immunity (Figure 54-2). At sites of infection and inflammation, bone marrow–derived progenitor cells respond to proliferative and differentiation signals. Granulocyte macrophage

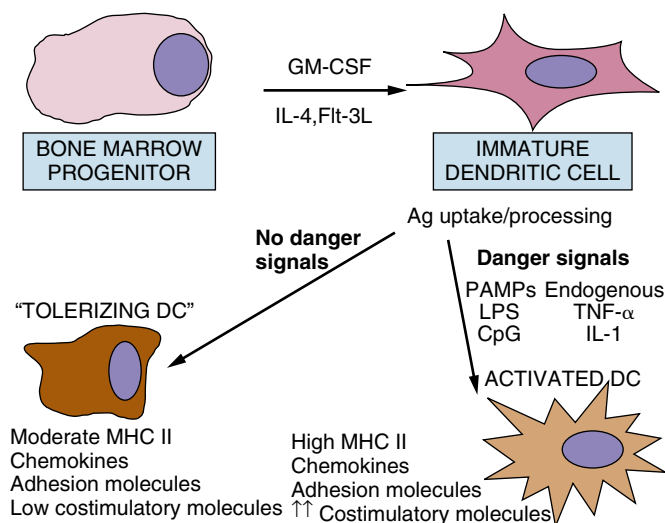


FIGURE 54-2 TWO PATHWAYS FOR DENDRITIC CELL (DC) ACTIVATION. DCs, the major antigen presenting cells (APCs) that regulate T-cell activation, develop from hematopoietic precursors under the control of various cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF). In the presence of activating signals from cytokines such as tumor necrosis factor (TNF) or exogenous pathogen-associated ligands for toll-like receptors (TLRs; such as lipopolysaccharides [LPS] or unmethylated CpG sequences), DCs become activated to express high levels of major histocompatibility complex (MHC) molecules, chemokines to attract T cells and costimulatory signals, including B7 family members, TNF family members, and proinflammatory cytokines (pathway on right). The result is activation of T cells specific for antigens presented by these DC. In the absence of DC activation signals, there is steady-state presentation of self-antigens by immature DC that do not provide adequate costimulatory signals for T cell activation (pathway on left). The result is tolerance induction among T cells specific for self-antigens presented by these unactivated DCs. Tumor-associated DCs are not fully activated and thus can induce tolerance among tumor-specific T cells.

colony-stimulating factor (GM-CSF), as well as other cytokines such as FLT-3L and interleukin-4 (IL-4), serve as mitogenic or comitogenic factors that induce an intermediate stage of DC differentiation, characterized by efficient antigen uptake and processing (46–50). Once they have ingested antigens at inflammatory sites in the tissue, immature DCs differentiate in response to a number of distinct “maturation” signals. Although many diverse molecules induce DC maturation, most appear to signal DCs via binding to two classes of receptor: the toll-like receptors (TLRs) and the tumor necrosis factor (TNF) receptor (TNFR) family. TLRs are “pattern-recognition receptors” (PRRs), which bind common chemical moieties expressed by pathogens termed “pathogen-associated molecular patterns” (PAMPs) such as lipopolysaccharide (LPS) and unmethylated CpG DNA sequences (51). The two best-characterized endogenous DC maturation factors are TNF- α itself and CD40L (52–54). In addition to TLRs, intracellular PRRs, including PKR, RIGI, MDA-5, and NOD1/2, recognize PAMPs from intracellular bacteria and viruses that invade the cytosol (55–57).

Maturation of DCs, which occurs as they traffic to draining lymph nodes, is characterized by transport of peptide–MHC complexes to the cell surface (58,59). In addition to provision of high densities of peptide–MHC complexes for T-cell stimulation (termed “signal 1”), DCs regulate T-cell activation and differentiation through provision of costimulatory signals in the form of cytokines, such as IL-12, and membrane-bound ligands of the B7 and TNF family (collectively termed “signal 2”). The ever-expanding panoply of costimulatory signals used by DCs to instruct T cells as to their pathway of differentiation and effector function defines a high degree of complexity to the communications that occur between APC and T cells. When immature DCs present antigens to T cells in the absence of costimulatory signals, the outcome is tolerance induction. This is a normal mechanism for maintenance of tolerance to self-antigens. It is also a mechanism by which tumors can induce immune tolerance to their own antigens (Figure 54-2). As discussed throughout this chapter, tumor-induced immune tolerance is a major barrier to successful vaccination of established cancers. Each of the molecular events involved in proliferation, antigen presentation, and costimulation represent potential targets that are being exploited in the design of immunotherapy approaches.

Basics of T-Cell Activation

Because virtually all of the signals to T cells begin at the cell membrane, vaccines and other immunotherapies can be enhanced by inclusion of ligands that bind these cell membrane receptors. The enhanced versatility of new vector systems allows for combinatorial construction of immunotherapies containing multiple elements that target multiple points in the pathway of T-cell activation.

Enhancement of Signal-1

In considering the immunogenicity of various vaccine formulations, it is commonly assumed that alterations in the immune response will depend strictly on the set of costimulatory signals

(signal 2) provided to T cells at the time of antigen recognition. However, it is now clear that both qualitative and quantitative characteristics of the peptide–MHC interaction with T-cell receptor (TCR; signal 1) are equally important in determining the outcome of T-cell responses. The two most well-defined parameters of TCR engagement are ligand density and TCR affinity. Low-affinity ligands result in partial agonist properties and ultimately in antagonist properties. The favored model to explain these findings is a kinetic proofreading model, which suggests that the TCR must be engaged by the peptide–MHC complex for a long enough time period to initiate the complete set of intracellular biochemical signaling events for T-cell activation (60). Even for high-affinity ligands, it has been demonstrated that exposure of T cells to ligand densities below activation threshold can also result in induction of T-cell unresponsiveness. A fundamental corollary of the immune tolerance hypothesis—that tumors arising endogenously are capable of inducing tolerance among T cells specific for neoantigens—is that the residual repertoire of tumor antigen–specific T cells will be low affinity or specific for epitopes presented at low density. Similar mechanisms may be operative when viruses evade immune elimination and set up a chronic carrier state.

Analysis of T-cell responses against defined tumor antigens has indeed provided experimental evidence for this notion. As mentioned previously, most melanoma-specific T cells that have been grown in culture appear to recognize melanocyte-specific differentiation antigens such as MART-1/melan-A, gp100, and tyrosinase (29–32). As specific MHC class I- and class II-restricted epitopes have been identified, a surprisingly large number appear to have extremely low affinities for their presenting MHC molecule, resulting in low-density peptide–MHC complexes on the tumor and on APCs loaded with the antigen (61). This low MHC affinity is generally associated with undesirable residues at critical anchor positions. In other cases, tumor peptides bind well to MHC molecules but available T cells display low affinities for the peptide–MHC complex.

As tumor antigens continue to be identified, there will be important opportunities to modify epitopes so that they are presented to T cells in a fashion that provides the most effective transmission of the TCR signal (signal 1). For antigens that have poor MHC binding affinity, a number of groups have demonstrated that alteration of MHC anchor residues to more favorable amino acids can result in dramatic enhancement of MHC binding while retaining the capacity for enhanced activation of T-cells specific for the original wild-type epitope (62,63). This results in a heteroclitic response in which vaccines containing the anchor-modified epitope can produce greater immune responses against the wild-type peptide in vivo resulting in enhanced antitumor immunity. Epitope engineering can also generate enhanced immunity at the level of TCR affinity for peptide–MHC. Thus, in the case of the low-affinity response to the GP70-derived peptide that is immunodominant in the murine CT26 colon cancer, a single amino-acid alteration did not affect MHC binding but increased the affinity of peptide–MHC for the T cell receptor by threefold. Immunization with DCs loaded with this altered peptide resulted in a dramatic enhancement in the expansion of T cells in vivo specific for the original wild-type peptide and enhancement of systemic antitumor immunity (64).

Peptide–MHC ligand density on APCs can be enhanced by targeting antigens into the MHC processing pathways. Two approaches have been used to accomplish this goal. One approach has used the linkage of targeting signals onto the antigen that will more effectively target them into MHC-processing compartments and pathways. Strategies for antigen targeting to the MHC class II processing pathway have used the invariant chain targeting signal and the endosomal/lysosomal targeting signal in the cytoplasmic tail of the lysosome-associated membrane protein-1 (LAMP-1). In the case of the LAMP-1 sorting signal, fusion genes of the E7 antigen linked to the LAMP-1 sorting signal resulted in increased targeting of E7 into the MHC class II processing pathway and resultant enhancement of presentation to E7-specific MHC class II-restricted CD4 T cells. Incorporation of the LAMP targeting signal onto the E7 gene enhanced CD4 responses and ultimate antitumor potency of both recombinant vaccinia and recombinant DNA vaccines (65–67).

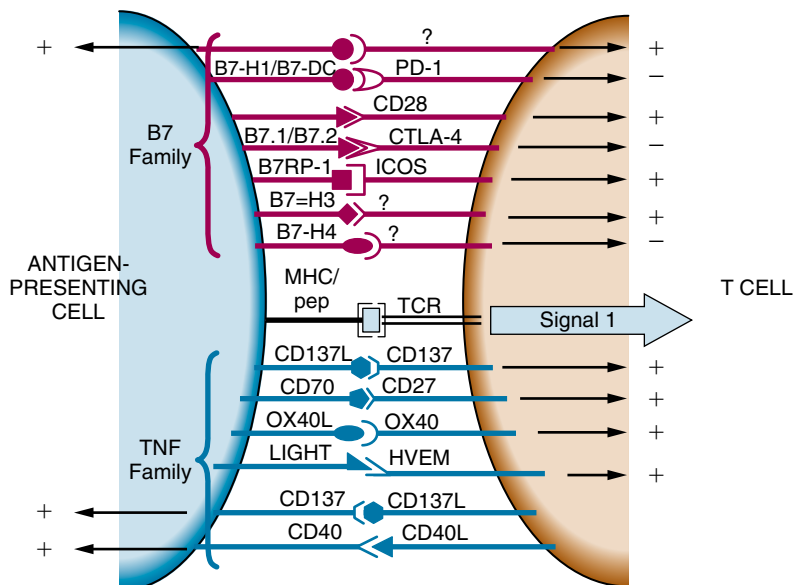
A number of MHC class I targeting approaches have also resulted in enhanced immunization potency. The antigen-hsp70 fusion genes described previously represent such an example that selectively enhances antigen-specific CD8 responses (68). Other validated targeting signals that enhance CD8 priming include calreticulin, VP22 (a herpesvirus-encoded protein) and the ER translocation subunit of *Pseudomonas* exotoxin (69,70). Another strategy for enhanced MHC class I processing has been the construction of “epitopes on a string” (71). This approach separates out individual epitopes from a given antigen and strings them together separated by linkers that encode basic amino acids that are good substrates for proteasome cleavage. As a number of these strategies likely function via distinct mechanisms, maximal loading of MHC on APCs will likely be achieved through combining different targeting signals.

Enhancement of Signal 2: Costimulation

Qualitative and quantitative elements of T-cell activation and differentiation are determined in large part by signals delivered by costimulatory molecules. As the number of costimulatory molecules increase, a picture is emerging in which T cell activation requires integration of a large number of different signals. The best-characterized costimulatory signals fall into three families: the B7 family, the TNF family, and cytokines (Figure 54-3).

Among these three categories of costimulatory molecules, the B7 family appears to be the only one that can signal unidirectionally from APC to T cell. Tremendous effort has been directed to engineering costimulatory molecules into vaccines and other immunotherapies in an attempt to enhance their activity. In the case of the B7 family members, most work has focused on B7.1 and B7.2. It will be interesting to see how the five new B7 family members (ICOS-L, B7h/B7RP-1, B7-H1/PDL-1, B7-DC/PDL-2, B7-H3, B7-H4) will fit into the armamentarium as they bind distinct receptors from B7.1 and B7.2 and have only partially overlapping biologic activity (72,73). Some of these B7 family members are purely costimulatory (i.e., ICOS-L), some are purely inhibitory (i.e., B7-H4), and some appear to be costimulatory or inhibitory (i.e., B7-DC). In the later case, variable costimulatory or inhibitory function is likely mediated

FIGURE 54-3 REGULATION OF T-CELL ACTIVATION. T-cell activation requires engagement of the T-cell receptor by major histocompatibility complex (MHC)–peptide complexes on the antigen-presenting cell (APC; signal 1) and is further regulated in quality and amplitude by multiple additional costimulatory and co-inhibitory signals, which are collectively referred to as signal 2. The major (but not exclusive) costimulatory signals come from members of the B7 family and tumor necrosis factor (TNF) family, each binding to cognate receptor(s) on T cells. In addition, T cells can provide activation signals to the antigen presenting cells, creating an amplification loop. Some B7 family members are co-inhibitory because their receptors transmit inhibitory signals and some (such as B7.1, B7.2) bind to both costimulatory (i.e., CD28) and coinhibitory (i.e., CTLA-4) receptors. In these cases, the co-inhibitory receptor is generally not expressed by naïve T cells; it is up-regulated on T-cell activation, thereby providing feedback inhibition.



by multiple receptors that transmit costimulatory or inhibitory signals. A second major category of costimulatory signals to T cells involves a set of ligand-receptor pairs in the TNF-TNF receptor family. These include CD40L/CD40, 4-1BBL/4-1BB, OX40L/OX-40, LIGHT/HVEM, and CD70/CD27 (74). As will be discussed in the following sections, preclinical studies suggest that the most potent immunotherapy approaches will likely involve combinations of vaccination and costimulatory agonists or blockers of inhibitory signals.

With these principles in hand, one can evaluate the complex nature of different vaccine approaches that have been developed over the years. Essentially all cancer vaccine strategies have been developed first in animal models and then translated into the clinic. It is commonly said that animal (particularly mouse) models do not predict human outcomes because many vaccine formulations that have appeared very effective in mice have not proved effective in humans. This is, however, an incorrect interpretation of the data. Commonly, vaccines have been employed in mouse tumor models in which tolerance has not developed or the tumor burden is very small or the tumor has not had time to organize its microenvironment. Most cancer patients that come to treatment have had their cancer for 1 to 5 years before it is detected. Newer mouse models of spontaneously arising endogenous cancer may offer much better opportunities to test vaccines and other adjunct immunotherapies in a manner more predictive of the human setting.

Whole-Cell Tumor Vaccines

Overview

Before the molecular identification of tumor-specific antigens, investigators used tumor cells themselves as a source of tumor antigen (Table 54-2). Efforts to modify tumor cells as vaccines date back roughly half a century. Whole-cell tumor vaccines have been generated through mixing with adjuvants aimed at enhancing

“immunogenicity” of tumor-specific or tumor-selective antigens incorporated therein (75–79). Another approach has been to modify whole tumor cells with chemicals such as dinitrophenol (80) or infect them with a virus (81). The general concept is that increasing the immunogenicity of tumor cells using adjuvants or expression of foreign antigens will enhance immune responses to the endogenous tumor antigens, thereby allowing the immune system to kill metastatic tumor deposits.

More recently, a new era in genetically engineered whole-cell vaccination has involved the modification of tumor cells through transfer of genes encoding cell membrane immunostimulatory molecules or cytokines. Although most of the clinical activity related to adjuvanted whole-cell vaccines is diminishing significantly, active clinical investigation continues for cytokine gene–modified whole-cell vaccines, particularly with the GM-CSF gene (described in the following sections). Adjuvanted whole-cell tumor vaccines have been tested extensively in patients with melanoma, renal cell carcinoma, and colorectal carcinoma. Most of these vaccine strategies have involved co-injection of autologous or allogeneic tumor cells with adjuvants such as BCG and *Cryptosporidium parvum* (75–79,82). While Bacille Calmette Guérin (BCG) and *C. parvum* were long known to represent reasonable vaccine adjuvants for generation of antibody responses, a limitation of this vaccination approach has been their relatively poor capacity to generate T-cell responses, particularly in the face of established tolerance. Initially, nonrandomized clinical trials were performed, which demonstrated hints of promise. In some of the studies that reported antitumor responses, the responses were shown to correlate with the return of delayed type hypersensitivity (DTH) responses to recall antigens and more importantly with the development DTH responses to autologous tumor cells.

Application of BCG-adjuvanted tumor cell vaccines to patients with bulky metastatic cancer demonstrated an insignificant clinical response rate. However, given the plethora of studies in animal models suggesting that cancer vaccination might be more effective in the setting of minimal residual disease, a number of studies using

Table 54-2 Different Forms of Cell Based Vaccines in Clinical Testing

Vaccine Type	Advantages	Disadvantages
Autologous Tumor + BCG/c. parvum	Polyvalent Incorporates all unique and shared tumor antigens	BCG, c. parvum not good adjuvants for generating strong CTL responses Many irrelevant “self” antigens included in vaccine; antigenicity uncharacterized Expensive, labor-intensive processing; consistency and standardization difficult to achieve
Allogeneic Tumor + BCG/c. parvum	Polyvalent Non-pt specific – standardized lots can be produced with cell lines	BCG, c. parvum not good adjuvants for generating strong CTL responses Many irrelevant “self” antigens included in vaccine; antigenicity uncharacterized Clinical effect depends on shared antigens between vaccine lines and patient’s tumor
Hapten Conjugated Autologous Tumor Vaccine	Polyvalent Incorporates all unique and shared tumor antigens	Hapten conjugation never shown to generate strong CTL responses Many irrelevant “self” antigens included in vaccine; antigenicity uncharacterized Expensive, labor-intensive processing; consistency to standardization difficult to achieve
Hapten Conjugated Allogeneic Tumor Vaccine	Polyvalent Non-pt specific – standardized lots can be produced with cell lines.	Hapten conjugation never shown to generate strong CTL responses Many irrelevant “self” antigens included in vaccine Clinical effect depends on shared antigens between vaccine lines and patient’s tumor
Adjuvanted Tumor Lysate (i.e., Melacine)	Polyvalent Non-pt specific – standardized lots can be produced with cell lines	Lysate not as effective as whole cells in generating T cell responses Many irrelevant “self” antigens included in vaccine Clinical effect depends on shared antigens between vaccine lines and patient’s tumor
Autologous gene transduced Tumor Vaccine (i.e., GM-CSF, termed autologous GVAX)	Polyvalent Incorporates all unique and shared tumor antigens Tremendous versatility in immune response activation depending on which gene or genes are transduced	Regulatory barriers associated with gene therapy Many irrelevant “self” antigens included in vaccine; antigenicity uncharacterized Expensive, labor-intensive processing; consistency to standardization difficult to achieve
Allogeneic gene transduced Tumor Vaccine (i.e., GM-CSF, termed allogeneic GVAX)	Polyvalent Tremendous versatility in immune response activation depending on which gene or genes are transduced Non-pt specific – standardized lots can be produced with cell lines	Regulatory barriers associated with gene therapy Many irrelevant “self” antigens included in vaccine Clinical effect depends on shared antigens between vaccine lines and patient’s tumor

BCG adjuvanted tumor vaccines clinical trials were undertaken in the minimal residual disease setting after resection of the primary tumor. Initial enthusiasm for a BCG adjuvanted, autologous colon cancer vaccine in patients with resected stage 2/3 colon cancer (83) as well as a melanoma vaccine consisting of a mixture of irradiated allogeneic human melanoma lines with BCG used in melanoma patients with stage 3 and resected stage 4 disease (84) was based on phase 2 studies and limited, single-institution phase 3 studies. The concern in the interpretation of clinical outcomes of these phase 2 studies is that it was unclear whether the untreated “historic controls” were truly comparable to the population of patients treated in the phase 2 studies. In the absence of careful case controlled comparisons, ultimate acceptance of these vaccines depended on pivotal randomized phase 3 studies in which both progression-free survival

and overall survival were the relevant clinical endpoints. In the case of the autologous BCG adjuvanted colon cancer vaccine, an initial randomized single institution study in The Netherlands claimed a longer overall survival in patients with stage 2 but not stage 3 colon cancer (83). Unfortunately, these findings were not reproduced in expanded multicenter trials, possibly owing in part to technical difficulties in consistent autologous tumor preparation as part of the patient-specific vaccine formulation (85). After 20 years of phase 1 and 2 studies with an allogeneic BCG adjuvanted melanoma vaccine, a randomized phase 3 clinical trial between BCG adjuvanted allogeneic melanoma cells versus BCG control demonstrated no evidence of enhanced overall survival for the BCG plus tumor vaccine arm (86). Although the phase 2 studies claimed to have demonstrated significant survival benefit relative to case-matched

controls, the case-matched controls demonstrated suspiciously short overall survival times relative to melanoma patients of similar stage from multiple other clinical studies. There were encouraging reports of responses to vaccination with BCG adjuvanted dinitrophenol (DNP) modified allogeneic melanoma vaccines (87). However, definitive randomized phase 3 trials have not been completed at the time of this writing. Although a number of these studies reported that patients with enhanced DTH responses after vaccination had better disease outcomes than patients who did not, these studies were largely devoid of analyses of antigen-specific T-cell responses, and it is unclear whether the association between DTH responses and enhanced survival had anything to do with the vaccination. A similar fate befell the melanoma vaccine Melacine, a mixture of lysates from multiple allogeneic melanoma cells admixed with the “detoxified” lipopolysaccharide derivative monophosphoryl lipid A (MPL) plus mycobacterial cell wall extracts. Despite encouraging reports from phase 2 studies, a definitive phase 3 study in patients with stage 2/3 operated melanoma failed to demonstrate a statistically significant effect on overall survival (88). A retrospective subset analysis suggested that HLA-A2⁺ and HLA-C1⁺ patients had greater benefit, but this result has not been confirmed in a prospective trial (89). A randomized trial of melacine plus low-dose interferon (IFN)- α 2b versus standard-dose IFN- α 2b alone demonstrated equivalence; however, given that IFN- α 2b is not reproducibly demonstrated effectiveness in the adjuvant treatment of stage 2/3 melanoma, the value of melacine plus low-dose IFN- α 2b remains in doubt (90).

One of the limitations of these trials is that none demonstrate definitive enhancement of T-cell responses against relevant antigens. In the case of melanoma, many tumor antigens recognizable by T cells are indeed well defined, and responses to them should be measured as part of the development process. As described previously, a more limited set of “immunorelevant” antigens are defined for other human cancers. In summary, the age of adjuvanted whole-cell or lysate tumor vaccines appears to be slowly drawing to a close and will likely be a historic footnote in the development of cancer immunotherapies.

Genetically Modified Tumor Vaccines

With the development of improved genetic techniques, emphasis has shifted to genetic modification of tumor cells to express immunostimulatory molecules. Building on the original studies of Lindenmann and Klein (91), who showed that vaccination with influenza virus-infected tumor cell lysates generated enhanced systemic immune responses against challenge with the original wild-type tumor, Fearon and colleagues used direct gene transfer to introduce the immunogenic influenza hemagglutinin (HA) gene into murine tumor cells to create genetically engineered vaccines (92). These HA transfectants induced a systemic immune response against challenge with the parental tumor. Gene transfer of viral antigens was eventually superseded with gene transfer of immune response-modulating genes. It is important to point out that although many of the strategies were designed with a specific mechanism in mind, it is becoming clear that genetic manipulation to

alter expression of even a single gene product can result in a complex cascade of cellular responses *in vivo* that ultimately may affect multiple aspects of antigen processing, presentation, and costimulation.

There are many ways to genetically modify tumor cells to augment T-cell-mediated antitumor immunity. One involves the genetic modification of tumor cells to express cytokines that function as attractants or differentiating agents for dedicated APCs such as DCs. Recruited DCs ingest released tumor antigens at the site of vaccination and present them together with appropriate costimulation required for the activation of a tumor-specific T-cell response. Alternatively, the tumor cell can be genetically modified such that it becomes the antigen presenting cell itself.

Both *ex vivo* and *in vivo* methods of gene delivery have been used in the development of genetically modified whole-cell cancer vaccines. *Ex vivo* gene delivery involves the modification of cultured cells. The genetically modified cells are subsequently administered to the host, typically after irradiation. Clearly, the most effective way to enhance expression of MHC molecules or to enhance expression of costimulatory molecules such as B7.1 or B7.2 is to genetically modify the tumor cell itself. However, when the goal is to deliver cytokines locally in a paracrine fashion, genetic modification of the tumor cells themselves is not necessary (93). A number of transduced bystander cytokine delivery systems have been developed (94). The efficacy of bystander cytokine delivery systems is comparable to that of direct gene modification of the tumor cell for augmenting antitumor immunity. It is however necessary that the transduced bystander cells are admixed with the tumor cells in an appropriate ratio. *In vivo* gene delivery involves the injection of a vector directly into the tumor mass that exists within the host (95) or uses systemic administration of a vector that selectively infects tumor cells or whose genetic material is controlled by a promoter that is only active in the tumor (96). *In vivo* gene delivery methods are generally much less reliable and reproducible in terms of efficiency and selectivity of gene expression within the tumor and have generally been limited to small clinical trials with no sustained development path.

Insertion of Genes Encoding Costimulatory B7 Family Members into Tumors

B7 molecules, originally described as activation antigens on B cells, are expressed on most APCs. The B7 family is now known to consist of more than seven different genes. Some of these genes encode molecules that are costimulatory, others are inhibitory, and some bind to both costimulatory and inhibitory receptors. The original B7 family members, B7.1 and B7.2, bind to 2 receptors: CD28 and CTLA-4 (97–99). CD28 is now well characterized as a critical costimulatory receptor for T-cell activation. Cross-linking of CD28 has been shown to enhance the level of lymphokine production by CD4⁺ T cells subsequent to antigen recognition. It also plays a role in costimulating CD8 cells. This enhanced lymphokine production appears to be due to enhanced transcription and enhanced messenger RNA stability. CTLA-4 is an inhibitory receptor that binds to B7.1 and B7.2 with higher affinity than CD28. Naïve T cells express CD28 but not CTLA-4. Once T cells are activated, CTLA-4 is up-regulated, providing for a feedback inhibition system to modulate the ultimate level of T-cell stimulation (100).

Transfection of B7.1 into some tumors results in rejection of that tumor, and in some cases, generates systemic immune responses capable of generating protection against challenges of the wild-type tumor at a distant site (101,102). Co-introduction of the B7.1 gene with other cytokines such as IL-7 or IL-4 or with genes encoding MHC class II molecules appears to result in enhanced generation of systemic immunity against the original tumor (103–105). It has been postulated that B7 introduced into tumor cells functions by enhancing the tumor's ability to directly co-stimulate T cells that recognize tumor antigens presented by that tumor's MHC molecules. However, there is additional evidence that B7-expressing tumors are better targets for NK cell killing, which subsequently results in enhanced release of tumor antigens with subsequent cross-presentation by host bone marrow–derived antigen presenting cells (106,107). More recently, tumors have been transduced with other members of the B7 family (see following sections). Transduction of tumor cells with B7-DC, a B7 family member predominately expressed on DCs (108), results in enhanced rejection of the transduced tumors with induction of systemic immunity (109). B7-DC, similarly to B7.1 and B7.2, binds to both as an undiscovered co-stimulatory receptor and the inhibitory receptor PD1. Transfection of tumors with a B7-DC mutant that costimulates but fails to bind PD1 results in greater antitumor immune responses than transduction of wild-type B7-DC (110). These studies demonstrate the profound capacity to generate genetically modified tumor vaccines with enhanced potency through engineering of specific sites within the transduced genes.

Introduction of Cytokine Genes into Tumor Cells

Genes that encode cytokines are the most common types of genes that have been introduced into tumor cells to generate genetically modified tumor vaccines (93). Tumor cells transduced with cytokine genes alter the local immunologic environment at the vaccine site, enhancing the presentation of tumor-specific antigens to the immune system or the activation of tumor-specific lymphocytes. Critically, the cytokine is produced at very high concentrations in the vicinity of the tumor, whereas systemic concentrations are relatively low. This paracrine physiology much more closely mimics the natural biology of cytokine action than does the systemic administration of recombinant cytokines. Since the initial reports of enhanced antitumor responses after vaccination with IL-2–transduced tumor vaccines (111, 112), many cytokine genes have been introduced into tumor cells with various effects on both tumorigenicity and immunogenicity. Some of these cytokines induce a local inflammatory response that results in elimination of the injected tumor. This local inflammatory response is most predominately dependent on components of innate immunity rather than the classic T cells. Ultimately, however, the most important outcome of vaccination is the generation of enhanced T-cell responses specific for the antigens expressed by the vaccinating tumor.

GM-CSF Gene-Transduced Tumor Vaccines

Among the vast array of cytokine gene transduced tumor vaccine studies, GM-CSF-transduced tumor vaccines have emerged as having shown significant evidence of clinical activity and are currently being tested in a number of phase 3 clinical trials. In the original study that identified GM-CSF, multiple cytokine, adhesion molecule, and costimulatory genes were introduced into the poorly immunogenic B16-F10 tumor using a replication-defective retroviral vector that produced consistent high levels of expression of each of the transgenes in the absence of selection, thereby eliminating variability caused by different levels of gene expression and resultant cytokine expression. Animals were vaccinated with the irradiated transductants, followed by challenge with unirradiated wild-type B16-F10 cells to doses 3 to 4 logs higher than the minimal tumoricidal dose (113). Although a number of cytokine genes in that study, such as IL-4 and IL-6, induced some measurable systemic antitumor immunity (114,115), the most potent systemic antitumor effect was produced by GM-CSF–transduced tumor cells. Many subsequent studies in other murine tumor models have validated the potent systemic immunity induced by GM-CSF–transduced tumor vaccines. Antitumor immunity induced by GM-CSF–transduced vaccines has been shown to depend on CD4⁺ and CD8⁺ T cells. In addition to the classic MHC class I–restricted CTLs, other effector arms mediated by CD4 cells have been shown to participate in the generation of maximal antitumor immunity. Th1 and Th2 effector arms have been delineated (116). The Th1 effector arm depends on IFN- γ and involves the activation of macrophages at sites of metastases to produce reactive nitrogen species (NO), as well as reactive oxygen species (superoxides). Eosinophils appear to be important Th2 effectors that are dependent on the production of cytokines such as IL-4 and IL-5 by tumor-specific CD4 cells. The presence of eosinophils at delayed-type hypersensitivity (DTH) sites in tumor metastases subsequent to vaccination with GM-CSF–transduced tumors is not only observed in animal models, but also has been a consistent observation in clinical trials with different tumor types (117).

GM-CSF–transduced vaccines (now commonly termed “GVAXs”) represent a prototypical example of paracrine cytokine adjuvants in which local production of a cytokine induces the differentiation of bone marrow–derived progenitor cells to activated APCs (118,119). In particular, in high enough doses, GM-CSF is an extremely potent differentiation factor for DCs. The three following findings all underscore the likely mechanism of action of GVAX vaccines as working through the differentiation of bone marrow–derived progenitors into DCs at the vaccine site followed by antigen uptake, processing, and traffic to drain lymph nodes:

1. Higher doses of GM-CSF lead to DC differentiation in vitro.
2. Maximal systemic immunity is only found with vaccines that produce high quantities of GM-CSF.
3. Increased DCs are found at GVAX vaccine sites as well as in lymph nodes draining GVAX vaccine sites (K. Joos, unpublished, July 2007).

Formal evidence that presentation of the tumor antigens to T cells is mediated by bone marrow–derived APCs for MHC class I– and MHC class II–restricted antigens has been obtained

through direct analysis of infiltrating DCs as well as bone marrow chimera models (120). These analyses demonstrate that T cells generated by the vaccine recognized antigenic epitopes presented exclusively by MHC alleles expressed by bone marrow–derived antigen presenting cells.

The discovery that tumor-specific T-cell responses are generated through cross-presentation has had fundamental implications for the application of GM-CSF gene–modified allogeneic vaccines. As described in the following paragraphs, significant limitations exist in the use of autologous GM-CSF gene–modified vaccines because of the difficulty in growing large numbers of autologous tumor cells *ex vivo* as well as the difficulties in transducing autologous tumor cells in a reproducible fashion to generate consistently high levels of GM-CSF production. Two discoveries in the early 1990s provided the rationale for the development of generic genetically modified allogeneic cancer vaccines that would obviate the need for individualized immunotherapy. First and foremost, the finding that some relevant immunologic targets are shared antigens (tissue specific or tumor associated) indicates that established cell lines for a particular tumor type may indeed contain “cross–reactive” antigens associated with that tumor type. Second, the finding that antigen presentation with cell-based vaccines occurs predominately, if not exclusively, through the cross-priming pathway by host bone marrow–derived APCs indicates that matching of HLA alleles between the vaccine cells and the host is unnecessary. A number of preclinical models have verified that as long as immunorelevant antigens are shared between vaccine and challenge tumor, complete MHC matching between the vaccine and the host does not interfere with priming of host-restricted immune responses capable of recognizing the challenge tumor (121,122). As described in the following sections, evidence for the generation of T-cell responses against tumor antigen epitopes presented by HLA alleles expressed by the patient but not by the GVAX vaccine cells has been derived in human vaccine trials of pancreatic cancer.

Clinical Development of GM-CSF–Transduced Tumor Vaccines

The single-vaccine approach being most actively tested in cancer patients is GVAX, with over 100 centers worldwide involved in many ongoing trials. Early clinical trials with GVAX cancer immunotherapies focused on autologous GVAX platforms in which individual tumors were resected, digested to single-cell suspensions, and genetically modified with various vector systems to secrete GM-CSF. The products were then irradiated to prevent further cell division and cryopreserved pending product release. Clinical use was restricted to the patient from whom the tumor cells were derived. Despite the technical and logistical challenges of such a labor-intensive approach, clinical results from these early trials demonstrated well-tolerated toxicity profiles, induction of immune responses, and encouraging signals of antitumor activity including radiologic tumor regressions.

The first autologous GVAX clinical studies to be performed were in renal cell carcinoma (117), metastatic melanoma (123), and prostate cancer (124). These early studies used standard

retroviral vectors for GM-CSF gene modification of the autologous tumor cells. Biopsies of intradermal sites of injection with GM-CSF gene–transduced vaccines contained distinctive macrophage, dendritic cell, eosinophil, neutrophil, and T-cell infiltrates similar to those observed in preclinical models of efficacy. Histologic analysis of DTH responses in patients vaccinated with GM-CSF–transduced vaccines demonstrated an intense eosinophil infiltrate that was not observed in patients who received nontransduced vaccines. An objective partial response was observed in a patient treated with GM-CSF gene–transduced vaccine who displayed the largest DTH conversion.

The most extensive evaluation of autologous GVAX immunotherapies has been performed in non-small cell lung cancer (NSCLC). A single institution phase 1 study was conducted in metastatic NSCLC using adenoviral-based GM-CSF gene transfer in an overnight process (125). DTH reactions were more common at dose level 2 or 3 (89%) than at dose level 1 (50%) as was evidence of inflammatory infiltrates in metastatic tumor biopsies, suggesting a dose response. Clinical activity was suggested by one patient with a mixed tumor response and two patients with isolated metastatic sites resected who remained free from disease recurrence for over 3.5 years. Based on these encouraging results a follow-up multicenter trial of this autologous, adenoviral-modified GVAX product was performed in NSCLC patients. Three of 33 advanced-stage patients had durable complete tumor responses following immunotherapy treatment.

Clinically, non–patient-specific GVAX platforms, which use GM-CSF–modified allogeneic tumor cells exclusively, offer numerous advantages over their autologous cell counterparts including manufacturing issues, consistency of vaccine, and on limitation on vaccine quantity. Scientific data from preclinical and clinical studies have provided support for the relevance of allogeneic GVAX immunotherapies and the seminal role of cross-presentation of allogeneic tumor-associated antigens by host APCs in the initiation of a cellular antitumor immune response. The one drawback of allogeneic GVAX vaccines is that unique, patient-specific tumor antigens are not targeted.

Several clinical trials of allogeneic GVAX immunotherapies are now under way, including two phase 3 trials in hormone-refractory prostate cancer that represent the most advanced program within the entire GVAX immunotherapy platform. The allogeneic GVAX platform has been evaluated in phase 1 and 2 trials in pancreatic (126), breast, and prostate (127) cancers and in a phase 1 study in chronic myeloid leukemia (CML). In the phase 1 study, 14 patients with resectable pancreatic cancer received four doses of immunotherapy in combination with surgical tumor resection and adjuvant 5-fluorouracil (5-FU)–based chemoradiotherapy. Immunotherapy was given at escalating doses from 10 to 500 × 10⁶ cells. One treatment was given postoperatively and an additional three monthly treatments were given after completion of adjuvant therapy. Autologous tumor DTH reactions were noted in three patients treated at doses of 100 to 500 × 10⁶ cells and all three have remained disease-free for greater than 5 years. Further analysis demonstrated induction of T-cell responses to the tumor-associated antigen, mesothelin, restricted to the HLA type of each responding patient (A2, A3,

and A24) that did not match the HLA type of either pancreatic cancer cell line used in the immunotherapy (128). These data provide direct evidence for induction of T-cell responses via cross-presentation of antigens expressed on allogeneic tumor cells by activated autologous APCs. Based on the encouraging results from this phase 1 trial, a phase 2 trial in 60 patients with resectable pancreatic cancer is under way. Preliminary results demonstrated encouraging survival data with an estimated 1-year survival of 88% and 2-year survival of 76%. A pilot study was performed using a GM-CSF-transduced CML line—K562/GM-CSF cells—as an allogeneic, non-patient-specific, GVAX immunotherapy for CML. In this study, 19 patients with chronic-phase CML with an incomplete molecular response to at least 1 year of imatinib, as measured by residual bcr-abl by reverse-transcriptase-polymerase chain reaction (RT-PCR), received K562/GM-CSF immunotherapy every 3 weeks for four treatments in combination with ongoing imatinib therapy. Further reductions in leukemia cell burden were demonstrated in ten of 19 patients with a complete molecular response in five patients (undetectable bcr-abl by RT-PCR) and a major molecular response in five (>1 log reduction in bcr-abl).

The most advanced clinical program using the GVAX platform of immunotherapies is an allogeneic prostate cancer vaccine approach using two prostate cancer lines, LNCaP and PC-3. Based on the favorable survival results seen in these two consecutive multicenter phase 2 trials (127), along with other evidence of clinical and immunologic activity, two multinational phase 3 trials, each in 600 patients, have been initiated. In the first trial, GVAX immunotherapy is being compared with docetaxel and prednisone in patients with asymptomatic Herceptin-resistant prostate cancer (HRPC). In the second trial, GVAX in combination with docetaxel is being compared with docetaxel and prednisone in patients with symptomatic HRPC.

Antigen-Specific Vaccines

The ultimate goal of cancer vaccine development is the use of antigen-specific vaccines that incorporate select tumor antigens into a vaccine vector(s) or adjuvanted formulation (Table 54-3). Antigen-specific vaccines have two intrinsic advantages over any type of cell-based vaccine. First, their formulation into a vaccine is much more versatile. Second, they do not contain the thousands of irrelevant or autoantigens included in a cell-based vaccines. The control over antigenic makeup afforded by antigen-specific vaccines is significant but requires knowledge of the best antigens to incorporate. The characteristics of an ideal tumor antigen include (1) highly selective expression by the tumor relative to normal tissues and expressed at reasonably high density on the surface of the tumor cell (as peptide-MHC complexes for T-cell recognition); (2) shared among most tumors of a particular type or ideally tumors with diverse histologies; (3) provides a growth advantage for the tumor, ideally required for tumor growth or survival; and (4) reasonable T-cell repertoire available in patients that has not been deleted and is not stringently tolerized. One of the major limitations in many of the antigen-specific vaccines tested clinically has been the application of antigens that do not meet these criteria.

Table 54-3 Different Types of Antigen Specific Cancer Vaccine in Clinical Testing

Vaccine Type	Advantages	Disadvantages
Peptide	Easy to produce	Poor immunogenicity HLA allele specific Can induce tolerance
Protein	Easy to produce	Poor immunogenicity Poor CTL induction
DNA	Easy to produce Versatile construction	Poor immunogenicity in humans
Virus	Good immunogenicity Versatile construction (some)	Safety (some viruses) Neutralizing immunity precludes revaccination Challenge to produce
Bacteria	Good immunogenicity Versatile construction Easy to produce Safety (antibiotics)	Regulatory hurdles

No one antigen may exist that does perfectly meet all these criteria. However, when planning to test a vaccine clinically, it is important to ask how well the candidate antigen(s) meets them. Ideally, antigen-specific vaccines should contain multiple “immunorelevant” antigens, particularly if they are not absolutely essential for tumor growth or survival. A final general principle is that a vaccine containing the best tumor antigen(s) will not enhance antigen-specific responses effectively if the vaccine vector or adjuvant is suboptimal. These principles will be evaluated in the following discussion on commonly studied antigen-specific vaccines.

Peptide Vaccines

The identification of T-cell-recognized tumor antigens at the peptide level spawned a major effort beginning in the 1990s to develop peptide vaccines (129–131). The fundamental concept of peptide vaccination is that minimal peptides—particularly MHC class I-restricted peptides that are recognized by CD8 killer cells—can efficiently load MHC molecules on the surface of cells without requiring internal antigen-processing routes. Early studies with peptide vaccines mixed with various adjuvants demonstrated induction of peptide-specific T cells in vivo and in some cases antitumor responses (132). Dramatic antitumor responses were reported using ex vivo-generated DCs loaded with specific tumor antigenic peptides (133). Ultimately however, despite ongoing murine studies and clinical trials, peptide vaccines have proved disappointing.

Clearly, one of the major advantages of peptide vaccines is that they represent the ultimate defined tumor antigen and therefore the capacity to monitor induction of T-cell responses to the immunizing peptide is optimal. However, there are a number of disadvantages associated with peptide vaccination. First, individual peptides are selective for specific MHC alleles and therefore cannot be used generically. This limitation has been circumvented through the use of mixtures of peptides that bind to common MHC alleles, thereby assuring that most patients will express at least one MHC allele that can present the peptide in the vaccine mix. Another

major issue with minimal peptides as vaccinating antigens is that they do not only load the MHC molecules of DCs that would activate immune responses, but will also bind to MHC molecules on the surface of cells other than DCs just as efficiently. The consequences of peptide presentation by these cells can be tolerance induction because they do not supply the appropriate costimulatory signals necessary for T-cell activation (134–136). Therefore it is possible that peptide vaccination could in fact be detrimental for immune responses. Indeed, Melief and colleagues have presented evidence in animal models that vaccination with long peptides that require processing is significantly superior to vaccination with minimal MHC-binding peptides (137). They have demonstrated that the advantage of vaccination with long peptides comes from the fact that only DCs can process long peptides, thus leading to selective antigen presentation by dendritic cells over other APCs that could induce tolerance.

Another major factor in peptide vaccinations is the adjuvant that is used. Peptides themselves are intrinsically nonimmunogenic and strong immunization with peptides in animal models is only observed when strong adjuvants capable of activating dendritic cells are mixed with the peptides. Peptides can also be loaded onto DCs grown *ex vivo* (see following sections) and reinjected into the patient. The most common formulation used for peptide vaccines in clinical trials is incomplete Freund adjuvant (IFA), an oil emulsion that does not contain any specific activators of DCs and is thus suboptimal. A few groups have reported enhanced immunogenicity of peptides conjugated to lipids (lipopeptide vaccines; 138,139).

Clinical trials with peptide vaccines in cancer have predominantly utilized HLA class I–restricted tumor antigen peptides, but some are including MHC class II–restricted peptides (140). The inclusion of MHC class II–restricted peptides can involve those derived from tumor antigens (such as tyrosinase in the case of melanoma) or peptides derived from foreign antigens that would nonspecifically stimulate CD4 helper cells that theoretically would provide help for enhanced stimulation of tumor-specific CD8 T cells responding to the tumor-specific MHC class I–restricted peptides.

Clinical trials have been performed using peptide vaccines for many different cancer types, although vaccination for melanoma is the most common clinical target of peptide vaccines. A number of clinical trials using peptides in the setting of bulky metastatic cancer or in the minimal residual disease setting have demonstrated induction of increased numbers of antigen-specific T cells using various methods with anecdotal clinical responses (141,142). Some methods use staining with peptide–MHC tetramers to directly visualize antigen specific T cells. Other methods such as enzyme-linked immunosorbent SPOT (ELISPOT) or intercellular cytokine staining (ICS) seek to measure induction of functional T cells through the productions of cytokines such as γ -interferon.

In none of the peptide vaccine trials (used either alone or with other agents) has significant clinical activity been demonstrated. Among the most interesting clinical results highlighting the dichotomy between induction of expanded numbers of peptide-specific T cells and the absence of clinical activity are the vaccine trials in melanoma that have used an anchor-modified gp100 peptide to generate enhanced binding to HLA-A2 (143). These

trials use repetitive vaccination with this peptide in IFA in patients with no evaluable disease (NED) after resection for stages 2 to 4 melanoma. In these trials, Rosenberg and colleagues demonstrated the capacity to induce tremendous expansion of antigen-specific CD8 T cells, in some patients reaching 50% of the total circulating CD8 T cells as measured by staining with peptide–MHC tetramers and peptide-induced γ -interferon production. Nonetheless, there was no evidence that relapse rate was significantly different from the relapse rate in the same group of melanoma patients not receiving vaccination. In some cases, relapsed melanomas could be demonstrated to have lost HLA-A2 expression or expression of the gp100 antigen, possibly representing an example of evasion or escape from the T-cell responses induced by vaccination. However, many of the relapsed tumors expressed HLA-A2 and gp100, thereby suggesting that the expanded populations of HLA-A2/gp100–specific T cells induced by peptide vaccination were ineffective at eliminating relapsing tumors. As more is learned about regulation of T-cell responses, it is quite plausible to imagine that T cells expanded in suboptimal conditions (i.e., in the absence of appropriate proinflammatory or costimulatory signals) could up-regulate expression of inhibitory molecules such as CTLA-4 or PD1 that would block them from developing the critical effector activity necessary to kill tumor cells. Thus, tumor antigen-specific cells could expand but not be effective against tumors.

Ex Vivo–Loaded Dendritic Cell Vaccines

The ability to culture DCs *ex vivo* has led to a plethora of studies of *ex vivo* antigen–loaded DCs as tumor vaccines. Although DCs can be loaded with lysates of tumor cells, they are typically loaded with peptides, recombinant protein, or transduced with various vectors or RNA-encoding specific antigens. Initially, it was demonstrated that loading of *ex vivo*–cultured DCs with either MHC class I–restricted peptides, whole proteins, or tumor lysates followed by administration back into the animal led to the generation of immune responses against the loaded antigen as well as antitumor responses (133,144–149). More recently, the advent of more efficient gene transfer vectors has led to approaches in which *ex vivo*–cultured DCs are transduced with genes encoding relevant viral or tumor antigens (150–152). A number of recombinant replication defective viruses have been used to transduce DCs. In addition, Gilboa and colleagues have demonstrated that purified RNA can be used to effectively transduce DCs with resultant presentation of encoded antigens (153). This strategy offers the interesting possibility that DCs could be transduced with the entire amplified transcriptome of a tumor cell, even when only tiny amounts of tumor tissue are available. The paucity of direct comparative studies leaves open the question of which method of loading DCs *ex vivo* will be the most effective. Another major issue with *ex vivo*–loaded DC vaccines is the degree of maturation that is induced *in vitro* and its relevance to homing and function of loaded DCs after reinjection. Maturation protocols used for DC vaccination are currently quite variable and range from monocyte conditioned medium to various defined agents such as TNF- α , IL-1, soluble CD40L, and prostaglandins (154,155). Concern has been raised that full-blown maturation/activation of DCs *ex vivo* to a stage normally achieved

once they are within paracortical regions of the lymph node will impair their ability to home to lymph nodes after reinjection. This has led to the suggestion that DCs should be loaded and reinjected in an immature state and allowed to mature *in vivo*. But such an approach has potential negative consequences as Steinman, Bhardwaj, and colleagues have demonstrated—immunization of patients with antigen-loaded immature DCs can actually result in tolerance or suppression of antigen-specific responses (156).

Elucidation of proliferative and maturation signals for DCs has led recently to approaches in which DCs are not only loaded with antigen but are transduced with genes encoding proliferation and maturation signals. This would result in autocrine DC stimulation *in vivo* after reinjection. In one study, DCs loaded with antigen were transduced with genes encoding GM-CSF and CD40L. These genetically modified DCs resulted in much more potent stimulation of antitumor immunity than immunization with DCs loaded with antigen alone (157).

Another approach aimed at providing DCs with a full complement of tumor antigens is the generation of DC–tumor-fusion vaccines (158,159). The concept behind this approach is to fuse autologous tumor cells with dendritic cells, thereby allowing the co-expression of all relevant tumor antigens together with all relevant DC molecules within the same cell. One of the major limitations to clinically translating an approach of this type is the efficiency with which fusion can be achieved between DCs and tumor cells in the absence of selection. Ultimately, it is critical that both preclinical and clinical DC vaccine studies identify the critical parameters of DC growth and maturation as well as antigen loading that result in therapeutically relevant levels of T-cell activation *in vivo*.

Many clinical trials with DC vaccines have been performed using DCs cultured and activated *in vitro* by various methods and loaded with tumor antigens of various types. As with most cancer vaccines, melanoma is the most common target, although other cancers have been targeted as well. Inductions of T-cell responses are commonly reported and interesting anecdotal clinical responses have been reported in phase 1/2 trials (160). One of the more interesting clinical DC vaccine approaches involves transduction of DCs with RNA-encoding telomerase that is targeted to the MHC class II processing pathway with the LAMP targeting signal (161). Telomerase is the enzyme that restores telomeres, the ends of chromosomes. Without telomerase, cells will eventually stop growing when their telomeres are exhausted. Tumors typically up-regulate telomerase making it a tumor-selective antigen. Vaccination with DCs transduced with telomerase–LAMP RNA led to significantly enhanced CD4 and CD8 responses specific for telomerase.

Two phase 3 clinical trials using DC vaccines have been negative, though one led to interesting politically motivated deliberations at the FDA. Schuler and colleagues compared temazdamide (DTIC) chemotherapy with peptide-loaded DC vaccination in stage 4 melanoma patients (162). Objective responses were low in both arms (<5%), and there was no statistically significant difference in overall survival. A retrospective subset analysis suggested that HLA-A2⁺/HLA-B44⁺ patients might derive greater benefit from vaccination, but this has not been verified in a prospective manner. The Dendreon Corporation has recently reported results of a small phase 3 trial

in patients with advanced prostate cancer comparing placebo with a DC vaccine prepared by crude enrichment of peripheral blood lymphocytes (PBL) followed by culture with a prostatic acid phosphatase–GM-CSF fusion protein (163). The primary endpoint of prolonged progression-free survival was not achieved, but continued evaluation of the patients demonstrated prolonged overall survival compared with placebo of 4.5 months that appeared statistically significant. The quality of this trial was questionable since the small size precluded careful matching of patient characteristics and registration was applied for on the basis of an endpoint different from that built into the original trial. In addition, initial evaluation of a follow-up phase 3 trial did not demonstrate even a significant trend toward improved overall survival in the DC vaccinated group. Although a majority of the advisory panel voted to approve the vaccine, the FDA ultimately chose to require additional supporting clinical data prior to approval (164). Additional supporting data have not been forthcoming as of this writing.

Heat shock Protein–Based Vaccines

Another interesting category of proteins that may target antigen effectively to DCs and furthermore into MHC processing pathways is that of the heat shock proteins (HSPs). It is now well established that complexing peptide antigens to certain HSPs such as gp96, hsp-70, calreticulin, and hsp-110 enhance their immunogenicity significantly (165–172). HSPs were first utilized as tumor vaccines by purifying them from tumor cells followed by immunization. HSPs isolated from tumors are naturally complexed with a whole array of tumor-associated peptides. Other approaches to link antigen to HSP have included the production of recombinant fusion proteins in which antigenic peptides are covalently or noncovalently linked to the HSP (173) as well as DNA-based vaccines in which fusion genes between antigen and HSP gene are incorporated. In one direct comparative study using the human papilloma virus (HPV)–E7 antigen as a model, it was demonstrated that DNA vaccines encoding an E7–hsp70 fusion gene were 30-fold more effective than the wild-type E7 gene in generating CD8⁺ responses (174). Immunogenic HSPs complexed with antigenic peptides have been shown to efficiently load the MHC class I processing pathway (so called *in vitro* cross-presentation; 175). Although the intracellular pathway by which heat shock proteins effectively load MHC class I molecules with their associated peptides has not yet been elucidated, Srivastava and colleagues have identified CD91, the α_2 macroglobulin receptor, as an important receptor for several heat shock protein (gp96, hsp-70, hsp-90; 176). Ultimately, the immunogenicity of HSPs has been proposed to result from their ability to activate APCs and target antigens to MHC processing pathways. One report has suggested that hsp-70 can activate macrophages via CD14/TLR-4 (LPS receptor)–dependent and –independent pathways (177). HSPs have also been reported to activate DCs (178) although the receptors that mediate these putative activation functions have yet to be elucidated.

Clinical trials with heat shock proteins have been ongoing. A phase 2 trial vaccinating women with premalignant high-grade cervical dysplasia caused by HPV-16 using a bacterial hsp 65–HPV16 E7 fusion protein demonstrated a 35% complete response (CR) with induction of E7-specific T-cell responses in roughly half of the patients; however, the cohort was too small to determine whether this response rate was statistically different from the ~25% spontaneous regression rate observed in this patient group without treatment (179). The only phase 3 trial with heat shock protein vaccines reported to date was in patients with operated stage 2/3 renal cancer, who were randomized to observation or treatment with autologous hsp96 purified from the resected primary tumor (180). This was a negative study in that no statistical difference was observed in relapse-free or overall survival. A second phase 3 trial of autologous hsp96 versus physician's choice (IL-2, resection, or chemotherapy) demonstrated no benefit relative to the physician's choice arm but found that patients receiving ten or more vaccine administrations had a longer overall survival than those who received lower numbers of vaccines.

The Growing Armamentarium of Vaccine Vectors

For all of the added value that recombinant DNA technology provides in engineering elements into vaccine constructs that enhance their potency, nature itself provides a virtually limitless array of delivery systems in the form of diverse microbes with potent intrinsic immunologic properties. These immunogenic properties derive from their expression of PAMPs, which activate DCs via TLRs and intracellular sensing pathways such as PKR, RIGI, and MDA-5; their ability to induce proinflammatory cytokine expression by infected cells; and their ability to target intracellular MHC processing compartments. Of the three major microbial classes—virus, bacterium, and fungi—viruses and bacteria have been the most intensively investigated. A few reports of engineered yeast vaccines emphasize the potential immunologic utility of the third microbial class.

Engineered Viruses

Viruses are the most diverse and efficient gene transfer agents whose natural cell tropism and biologic features can significantly enhance the immunogenicity of antigens carried within them (Table 54-1). Using standard recombination approaches, Moss and Paoletti were the first to explore recombinant viruses as vaccine vectors. They used vaccinia virus, a highly immunogenic virus related to smallpox that is relatively nonvirulent in immunocompetent individuals. In most cases, a single immunization with recombinant vaccinia carrying a gene expressing an antigen will generate significantly greater immune responses against that antigen than the corresponding protein or peptide epitopes mixed with standard adjuvants (181–183). This is particularly true for CTL generation. To date, many viruses have been explored as recombinant vaccine vectors, including attenuated replication-deficient poxviruses (such as modified vaccinia anca, fowlpox, and canarypox),

adenovirus, herpesviruses and Venezuelan equine encephalitis virus (184–187). Each of these viruses has various advantages and disadvantages and no clear “winner” has emerged as the absolute vector of choice. Features of viruses that can enhance their potency as vaccine vectors include their ability to induce immunologic “danger” signals at sites of infection and to directly infect APCs. Features of viruses that can diminish their potency as vaccine vectors include the presence of virally encoded inhibitors of immunity. These include molecules that block processing and presentation in the MHC class I pathway (such as TAP inhibitors and inhibitors of MHC class I traffic out of the endoplasmic reticulum) and cytokine decoys, to mention a few (188). Deleting immunologic inhibitory genes from recombinant viruses may further enhance their vaccine potency while attenuating their virulence.

A major barrier to virus-based vaccination are neutralizing antibodies in pre-exposed or prevaccinated individuals that inhibit the initial round of infection and replication, thereby quenching their ability to immunize. Individuals who have never been previously exposed to the vaccinating virus generate neutralizing antibody after the first vaccination, thereby precluding subsequent vaccination with the same vector. This finding has led to the concept of cycling different viral vectors in “prime-boost” formats. Dramatic enhancement of immunization potency has been observed in prime-boost formats between different viruses such as vaccinia followed by fowlpox between DNA vaccines and recombinant viral vaccines (189,190).

Among the large number of clinical trials with viral vaccine vectors, the most extensive have involved poxvirus vectors. Based on enthusiasm from preclinical experiments, a number of prime-boost studies have been performed using vaccinia followed by fowlpox (191). A phase 3 study in patients with inoperable pancreatic cancer was performed using a vaccinia-fowlpox prime-boost schedule versus chemotherapy or supportive care. The viral vectors incorporated two antigens—carcinoembryonic antigen and MUC-1—and included ICAM-1, LFA-3, and B7.1 genes to putatively enhance the costimulatory activity of infected DCs (although this has never been proven). The trial was negative. Phase 2 trials with a similar prime-boost regimen for advanced prostate cancer using PSA as the antigen have provided interesting results but clinical benefit has not been definitively demonstrated. Regulatory hurdles and the inability to vaccinate repetitively are likely to preclude further development efforts for viral vaccines in cancer.

Engineered Bacteria

Genetic engineering of intracellular bacteria such as BCG, *Salmonella*, *Shigella*, and *Listeria* has produced a number of interesting and promising vaccines (192–195). In principle, bacteria that enter APCs may represent a good vehicle for delivery of recombinant antigens. In certain cases, such as *Listeria*, the bacteria exhibit complex life cycles that involve both phagolysosomal and cytoplasmic stages. Thus, recombinant *Listeria monocytogenes* (LM) engineered to secrete antigens will load the MHC class II–processing pathway during the phagolysosomal phase and the MHC class I pathway during the cytosolic phase of the life cycle. In addition, a number of recombinant bacteria actively induce

infected APCs to secrete proinflammatory cytokines such as IL-12. More recently, recombinant bacteria have been used as vectors for delivery of DNA vaccines (196–198). Thus, bacterial vaccines containing plasmids with eukaryotic promoter and enhancer elements driving the antigen gene result in potent immunization. These results indicate that the bacteria can directly transfer plasmids into eukaryotic transcriptional compartments within infected APCs.

The LM vectors are among the most promising bacterial vectors being developed for therapeutic vaccination of cancer. Dubensky and colleagues have identified a number of approaches to dramatically attenuate the virulence of LM without diminishing its immunogenicity. One approach is to knockout the ActA and Internalin(Inl)B genes of LM (199). Knockout of ActA does not prevent the initial infection of cells with LM, but eliminates the capacity for cell-to-cell spread necessary for propagation of LM infections. Knockout of the InlB gene eliminates the capacity of LM to infect hepatocytes while not affecting the capacity of LM to infect APCs. Thus, infection with InlB mutant LM generates strong intrahepatic inflammatory responses with minimal destruction of hepatocytes. ActA/InlB double-mutant LM are equivalently immunogenic to wild-type LM, but are 4 to 5 logs attenuated in their virulence. Another approach to virulence attenuation of LM (that is applicable to other bacterial vectors as well) involves the knock-out of DNA repair genes together with limited DNA cross-linking using psoralen derivatives. Because the DNA repair system has been knocked out, bacteria can be inhibited from replicating themselves with as few as a single DNA cross-link per bacterial genome. This approach maintains metabolic activity while formally “killing” the bacteria. These killed but metabolically active (KBMA) organisms maintain significant immunogenicity, but have highly attenuated virulence (200).

Enhancement of Antitumor Immune Responses Through Inhibition or Elimination of Regulatory T Cells

Over the past 10 years, regulatory T cells (T_{reg} s) have emerged as a central player in maintenance of the tolerant state as well as general down-regulation of immune responses to pathogens (201,202). Not surprisingly, they appear to play a role in tolerance to tumor antigens as well as the resistance of tumors to immune-mediated elimination (203,204). $CD4^+$ regulatory T cells are characterized by expression of a central master regulatory transcription factor—FoxP3—whose role in the gene expression programs of regulatory T cells is being actively studied (205). While $CD4^+$ regulatory T cells selectively (but not specifically) express a number cell membrane molecules, including CD25, neuropilin, GITR, and LAG3 (201,206–208), their overall genetic program and inhibitory capacity are dependent on sustained expression of FoxP3 (209,210). Mechanisms of immune suppression by regulatory T cells vary and include production of inhibitory cytokines such as IL-10 and TGF- β (211–213). In keeping with the emerging appreciation that tumors are by nature highly tolerogenic,

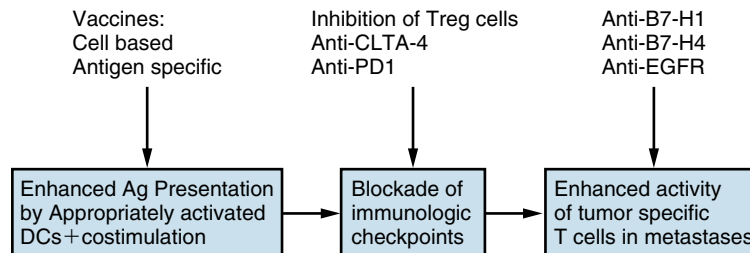
numerous murine studies have demonstrated that T_{reg} s expand in animals with cancer and significantly limit the potency of antitumor immune responses, natural or vaccine induced. For example, in a study by Suttmuller et al. (214), a combination of GM-CSF-transduced tumor vaccine plus anti-CTLA-4 antibodies was much more effective at eliminating established tumors when animals were treated with anti-IL-2 receptor α antibodies to eliminate $CD4^+$ regulatory T cells (214). It is now appreciated that treatment with low-dose cyclosporin is a relatively simple and reasonably effective way to temporarily eliminate cycling regulatory T cells (215–218). This appears to be a major mechanism by which pretreatment with low-dose cyclosporin prior to vaccination can significantly enhance the capacity of vaccines to break tolerance. As new cell membrane molecules that define regulatory T cells are identified, the capacity to block regulatory T-cell activity with antibodies to these molecules presents new opportunities for immunotherapeutic strategies to break tolerance to tumor antigens. It has been proposed that a major mechanism for enhancement of immune responses with anti-CTLA-4 antibodies (see following section) was through depletion of or a direct inhibitory effect on T_{reg} , which expresses significant levels of CTLA-4. However, a number of lines of evidence suggest that CTLA-4 blockade does not deplete T_{reg} numbers, at least in the peripheral blood, and has its major effect on effector cells.

Despite the interest in T_{reg} depletion to enhance vaccine potency, few clinical reagents exist to accomplish this other than cyclosporin. One reagent, ONTAK—an IL-2/diphtheria toxin conjugate approved for the treatment of HLT-1-induced leukemia—has been tested clinically in conjunction with an RNA-transduced DC vaccine in patients with renal cancer (219). Modest decreases in circulating peripheral $CD25^+$ T_{reg} s were reported along with enhanced CTL responses relative to patients treated with the DC vaccine alone. The capacity to maintain depletion T_{reg} through CD25 targeting has been questioned by other groups (220).

Enhancement of Cancer Vaccine Potency with Monoclonal Antibodies That Enhance Costimulation or Block Immunologic Checkpoints

The pessimism about the prospects for cancer vaccines engendered by the many failures of definitive phase 3 trials is balanced with the emerging enthusiasm about agents that can synergize with vaccines through enhancement of costimulation or blockade of immune checkpoints, including inhibitory signals that are up-regulated within the tumor microenvironment that block the effector activity of activated T cells (Figure 54-4). As described previously, maximal activation of T cells requires not only effective engagement of their TCR (signal 1) but also the appropriate level of costimulatory signals (signal 2). In addition to proinflammatory cytokines, the major costimulatory molecules include members of the B7 family, acting through costimulatory receptors on T cells, and TNF family members, acting through cognate TNF

FIGURE 54-4 COMBINATORIAL APPROACHES TO IMMUNOTHERAPY. Preclinical and preliminary clinical experience suggests that the most effective immunotherapy will combine vaccination with agents that inhibit immune checkpoints that down-modulate the amplitude of T cell responses (such as CTLA-4), maintain T-cell tolerance (such as T-regulatory cells), and inhibit effector immune responses in the tumor microenvironment (such as B7-H1 and B7-H4). Many of these inhibitory signals can be blocked with antibodies against the inhibitory receptor.



receptor family members expressed on T cells. Some of the costimulatory receptors, such as CD28 (costimulatory receptor for B7.1 and B7.2) are expressed on naïve or resting T cells whereas others (such as CD137/4–1BB) are only expressed on activated T cells and thus serve a role in amplifying T-cell responses. Another subgroup of costimulatory receptors includes those expressed on APCs. CD40 is the prototypical example and transmits activating signals from T cells, which express CD40 ligand, to B cells and DCs. A number of agonistic antibodies for costimulatory receptors have been produced and some of these indeed enhance the efficacy of vaccines in preclinical models in addition to demonstrating efficacy as single agents.

The second category of signals that regulate T-cell responses are inhibitory signals commonly referred to as immune checkpoints. These can be divided into feedback inhibitory signals expressed during the period of initial T-cell activation and signals that are delivered in the peripheral tissues to down-modulate the destructive effects of activated T cells. CTLA-4, the inhibitory counter-receptor for B7.1 and B7.2, is thought of as the index example of a negative-feedback receptor to modulate the level of initial T cell activation (221,222) whereas PD1, the receptor for B7-H1 and B7-DC, is thought of as an activation induced receptor to down-regulate effector T cell responses in the periphery (223–227). In reality, these functional distinctions are not nearly as clear, nor is the overlapping versus nonoverlapping function between these receptors well defined at this time. However, it is clear that antibodies that block immune checkpoints (directed against ligand or receptor) can have profound enhancing effects on vaccines (228–232).

In this final section, we cover receptors and ligands for which antibodies have been developed that enhance the efficacy of cancer vaccines in preclinical models. Ultimately, if therapeutic vaccines are to have their place in the repertoire of anticancer therapies, it will be in combination with agents such as those described in this

section as well as inhibition of T_{reg} cells as described in the preceding sections. Unfortunately, there is little clinical work at this time on synergy between vaccines and costimulatory agonists and checkpoint antagonists, largely because it is the paradigm in oncology drug development to initially develop therapies as single agents prior to developing combinations.

Summary

The failure of cancer vaccines to generate defined clinical efficacy in randomized phase 3 trials has brought to the fore the question of whether T-cell responses potent enough to be clinically meaningful can be generated. Although not feasible for general use, adoptive T-cell transfer studies with tumor-reactive T cells grown *ex vivo* suggest that antitumor efficacy is indeed achievable if enough T cells with the appropriate effector activity can be generated. Achieving this *in vivo* with vaccination remains a challenge because tumor-specific T cells from cancer-bearing patients exist in a hypoactive state, and tumors possess multiple resistance mechanisms. Most clinical cancer vaccine trials have been limited by a number of deficiencies, including vaccine formulations that do not effectively activate DCs, poor choice of antigen (for antigen-specific vaccines), poor choice of patient population (advanced metastatic cancer), inability to repetitively vaccinate (viral vaccines that generate neutralizing humoral immunity after one administration), and failure to combine vaccination with other agents that could enhance vaccine potency. Ultimate establishment of vaccination as a useful cancer therapy requires that these limitations be addressed. Most important, combinatorial approaches that enhance the efficacy of vaccines using agents that enhance T-cell activation status via costimulatory agonists and checkpoint antagonists will maximize the therapeutic potential of cancer vaccines.

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Monoclonal Antibody Therapy of Cancer

The development of monoclonal antibody therapy of cancer represents one of the major achievements and application of molecular medicine. We describe the structure and function of these biologic drugs, how they are being used clinically at the present time, and some of the new agents and applications of established antibodies that are undergoing clinical evaluation.

Background

To understand the origin and structure of monoclonal antibodies, we need to review vertebrate adaptive immunity. The immune response to foreign antigens includes the selection and production of immunoglobulin proteins by host clonal B lymphocytes. The most prevalent immunoglobulin molecule (IgG) consists of two identical heavy chains (50 kD) and two identical light chains (25 kD; [Figure 55-1](#)). Heavy chains have one variable domain (VH) and three constant domains (CH1, CH2, and CH3). Light chains contain one variable domain (VL) and one constant domain (CL). The amino-terminal ends of the VH and VL domains bind antigen. These domains have two antiparallel β -sheets with an intermolecular disulfide bond. Three loops at the end of the β -sheets form the hypervariable antigen recognition sites and are called complementarity determining regions (CDRs). The rest of the variable domains are the scaffold and are called framework regions (FRs). There are six CDRs per antibody arm (two identical arms per IgG) that form the antibody binding pocket ([Figure 55-2](#)). The C-terminal end of the heavy chains (CH2 and CH3) is called the Fc portion and mediates antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity and serum half-life. Each clonal B cell produces a unique immunoglobulin or antibody. Thus, the host with millions of reactive B lymphocytes generates a polyclonal response to infectious organisms.

In 1975, Kohler and Milstein immunized mice with non-murine cells (sheep red cells), isolated the mouse splenocytes (murine B cells) and then used a waxy compound (polyethylene glycol) to fuse together the B cells with a drug-marked (hypoxanthine-guanine phosphoribosyltransferase [HGPRT] deficient), immortal murine plasma cell ([Figure 55-3; 1](#)). The technique yielded hybridomas

that grew under conditions (hypoxanthine/aminopterin/thymidine media) where the parent B cells and myeloma cells died. By limiting dilution cloning, hybridomas producing single immunoglobulin or antibody molecules were obtained. With proper immunizations and in vitro screening methods, hybridoma monoclonal antibodies reactive with human tumor antigens were made ([2](#)). Large quantities of these monoclonal antibodies were prepared for clinical studies aided by advances in biotechnology including genetic engineering and bioreactors. Early clinical studies with murine monoclonal antibodies gave encouraging results, but the murine backbone was recognized as foreign by the human immune surveillance. This property led to human antimurine Ig antibody (HAMA) production and rapid clearance of the drug. Additionally, the murine Fc domains were inefficient at human effector functions (described in subsequent sections). A series of breakthroughs led to creation of mouse/human chimeric antibodies in which mouse variable regions were linked to human constant regions, then genetically engineered “humanized” antibodies in which CDRs were grafted onto variable domain frameworks. Finally, fully human antibodies were produced by either screening human antibody fragments from bacteria or phage display or by immunization of transgenic mice bearing the

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FIGURE 55-1 Immunoglobulin G antibody domains as described in text. (From Kim SJ, Park Y, Hong HJ. Antibody engineering for the development of therapeutic antibodies. *Mol Cells* 2005;20:17–29.)

FIGURE 55-2 Alpha-carbon backbone of antibody Fab region with VH and VL with β -sheets shown as flat arrows and loops shown as tubes. Antigen-binding sites formed by residues of loops at top of figure. (From Kim SJ, Park Y, Hong HJ. *Antibody engineering for the development of therapeutic antibodies*. Mol Cells 2005;20:17–29.)

human immunoglobulin gene repertoire (Figure 55-4; 3,4). These improved monoclonal antibodies were found to be less immunogenic and more stable and active in patients.

Antibodies can destroy cancer cells by at least three mechanisms. In the first method, the antibody binds or blocks a ligand-receptor signaling pathway critical to tumor cell survival. The antibody may bind the ligand itself (i.e., vascular endothelial growth factor for bevacizumab) or the receptor (i.e., epidermal growth factor family members for cetuximab and trastuzumab and calcium

channel for rituximab). In the second method, the antibody binds the tumor cell surface and recruits host effector mechanisms including complement and antibody-dependent, cell-mediated cytotoxicity (ADCC; e.g., rituximab and alemtuzumab) or binds effector cells and overcomes tumor-mediated tolerance (e.g., CP-870,893, ticilimumab and ipilimumab). Finally, the antibody can be chemically conjugated to a radioisotope (^{90}Y -ibritumomab tiuxetan and ^{131}I -totuzumab), cytotoxic compound (gemtuzumab ogozamicin), or toxin (BL22).

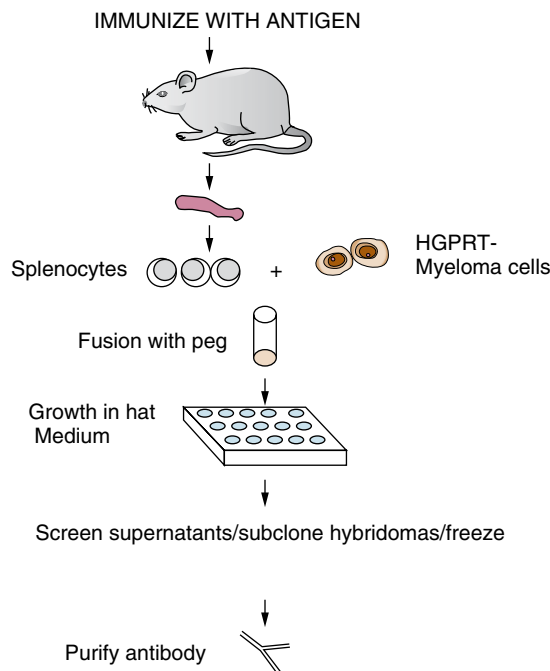


FIGURE 55-3 Schema for hybridoma monoclonal antibody generation. Steps include animal immunization and boosting, splenocyte isolation, fusion with HGPRT (hypoxanthine-guanine phosphoribosyltransferase) minus myeloma cells, aliquoting and growth in HAT medium, screening well supernatants for particular antibody, subcloning, expansion, and freezing.

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FIGURE 55-4 Antibody engineering for humanization. (From Kim SJ, Park Y, Hong HJ. *Antibody engineering for the development of therapeutic antibodies*. Mol Cells 2005;20: 17–29.)

Table 55-1 FDA-Approved Anticancer Antibodies

Antibody	Type	Antigen	Disease	Toxicity
Rituximab	C	CD20	B-cell lymphoma	Infusion reaction
Alemtuzumab	H	CD52	CLL	Infections
Trastuzumab	H	HER2	Breast cancer	Heart failure
Bevacizumab	H	VEGF	Colon cancer	Hypertension
Cetuximab	C	EGFR	Colon cancer	Acne rash
Gemtuzumab ozogamicin	H/D	CD33	AML	Liver failure
⁹⁰ Y-ibritumomab tiuxetan	M/R	CD20	B-cell lymphoma	Myelosuppression
¹³¹ I-tositumomab	M/R	CD20	B-cell lymphoma	Myelosuppression, hypothyroidism

AML, acute myelogenous leukemia; C, chimeric; CLL, chronic lymphogenous leukemia; D, drug conjugate; FDA, U.S. Food and Drug Administration; H, humanized; M, murine; R, radionuclide conjugate.

Approved Antibody Compounds

Eight monoclonal antibodies or antibody conjugates have been approved in the United States for use in human cancers (Table 55-1). Each of these antibodies or antibody conjugates has produced dramatic and durable responses with tolerable side effects. Because of the diversity of conditions such as target antigens and agent compositions, the optimal applications and side effects are different for each. Consequently, we will discuss them individually.

Rituximab is a chimeric mouse/human IgG1 antibody reactive with the human CD20 antigen present on all normal and most malignant B cells (5). CD20 is a 35-kD phosphoprotein calcium channel. Repeated weekly treatments with 375 mg/m², rituximab yielded a 50% response rate in relapsed low-grade follicular non-Hodgkin lymphoma (NHL) with a median time to progression of 1 year (6). Subsequent work demonstrated an improved response rate in large cell and follicular NHL with upfront therapy combining rituximab with CHOP chemotherapy (7,8). Other B-cell lymphomas are also responsive to rituximab including Waldenström macroglobulinemia, post-transplant lymphoproliferative disease, mantle cell lymphoma, Burkitt lymphoma, and chronic lymphocytic leukemia (9–13). Rituximab clinical resistance is associated with allelic polymorphisms in the FcγRIIIa receptor (14), up-regulation of complement resistance proteins CD55 and CD59 (15), and higher lymphoid tissue gene expression of genes involved in complement, cytokines, T-cell, and tumor necrosis factor signaling (16). Thus, rituximab may kill cells by recruiting host immune effectors. Side effects are remarkably minimal (17). Infusion-related reactions occur within the first few hours of usually the first infusion with chills, fever, nausea, fatigue, headache, pruritis, and a sensation of throat swelling. While long-term (6 months) reductions in normal B-cell populations are seen, immunoglobulin levels are maintained and infections are rare.

Alemtuzumab is a humanized IgG1 targeting the CD52 antigen expressed on most normal and malignant lymphocytes and monocytes (18). CD52 is a 21-kD glycoposphatidylinositol-linked glycoprotein. In animal models, congeners of alemtuzumab induce cell death by ADCC and complement activation (19).

Alemtuzumab produces responses in 40% and 90% of heavily pretreated and previously untreated chronic lymphocytic leukemia (CLL) patients, respectively (20). Disease in blood and bone marrow responded more frequently than nodal disease. Some responses were durable lasting over 2 years. Responses were also seen in T-cell lymphoma and HTLV-1-associated adult T-cell leukemia patients (21). Side effects were of two types. First, an infusion reaction with fever, rigors, rash, nausea, vomiting, and dyspnea was seen. Second, prolonged depression of normal T- and B-cell levels led to opportunistic infections including reactivation of cytomegalovirus (CMV) and fungal infections (22).

Trastuzumab is a humanized IgG1 antibody which binds the HER-2 member of the epidermal growth factor receptor family overexpressed on a fourth of breast cancers (23). HER-2 is a 185-kD tyrosine kinase receptor that lacks its own ligand-binding domain. Binding of trastuzumab to the extracellular domain of HER-2 down-regulates HER-2 cell surface receptor and inhibits interaction with HER-3, leading to reduced PI3K and phospho-AKT apoptosis inhibition (24). In fluorescence in situ hybridization (FISH)-positive metastatic breast carcinomas, the single agent response rate was 30% and the combination chemotherapy with trastuzumab response rate was 60% (25). Benefit in the setting of adjuvant combination therapy and neoadjuvant therapy in locally advanced breast cancer has been demonstrated (26,27). Duration of response is months to a year. Side effects include an infusion reaction with fever, chills, fatigue, and a late cardiotoxicity mediated by HER-2 present on cardiomyocytes (28).

Bevacizumab is a humanized antibody that binds vascular endothelial growth factor (VEGF) and blocks VEGF binding to the VEGF receptor on tumor blood vessel (29). Single-agent bevacizumab produced few responses but prolonged disease-free survival of renal cell carcinoma patients compared with controls (30). When combined with chemotherapy, bevacizumab has increased the response rate and response duration in metastatic colon carcinoma and non-small cell lung cancer (31–34). Side effects are limited to hypertension, proteinuria, and bleeding. Impaired healing and perforations have been observed in phase 3 trials.

Cetuximab is a humanized monoclonal antibody reactive with the extracellular domain of the HER1, epidermal growth factor receptor (EGFR). Cetuximab blocks EGFR activation (35). Although immunohistochemical measurement of EGFR in tumors does not correlate with response (36), the level of phospho-EGFR and EGFR gene copy number does predict clinical benefit (37,38). Furthermore, activation of the bypass pathway, mutant K-Ras, is associated with cetuximab resistance (38). Thus, only some tumors may be dependent on EGFR for growth and survival. Further, EGFR-dependent tumors show cell cycle arrest, apoptosis, and inhibition of angiogenesis mediated by hypoxia-inducible factor-1 α (39). Although the single-agent response rate in chemotherapy-refractory colorectal cancer was 9%, in combination with irinotecan, the response rate rose to 24% (40,41). Combination of cetuximab with radiotherapy doubled the duration of progression-free survival and overall survival in patients with squamous cell carcinoma of the head and neck compared with radiotherapy alone (42). For pancreatic cancer patients, gemcitabine combined with cetuximab yielded better progression-free and overall survival than historical trials with gemcitabine alone (43). Side effects include an acneiform rash, asthenia, and fatigue. The rash is associated with higher HER1 dimerization in skin (44). Topical vitamin K3 prevents EGFR inhibition from cetuximab in the skin in animals and may be useful in patients.

Gemtuzumab ozogamicin (GO) is a conjugate of a humanized anti-CD33 antibody with the cytotoxic drug, calicheamicin (45). GO binds to CD33 antigen on leukemic blasts, internalizes, and undergoes disulfide bond cleavage. Calicheamicin then enters the nucleus and triggers double-strand DNA breaks. Cell death follows. Resistance may be due to absence of cell surface CD33, excess anti-apoptotic proteins or membrane transporter-mediated drug efflux (46,47). Twenty-five percent of relapsed acute myeloid leukemia (AML) patients respond with remissions lasting only 6 months (48). Similar response rates and response durations are seen in *de novo* elderly AML patients (49). Toxicities include myelosuppression and veno-occlusive disease (50).

⁹⁰Y-ibritumomab tiuxetan consists of an anti-CD20 murine monoclonal antibody covalently linked to the β particle emitting radionuclide ⁹⁰Y (51). Yttrium radiometal is attached via a macrocyclic chelate. The high-energy β -emitting particles from ⁹⁰Y have a mean path length of 3 mm and deposit energy adequate to kill the targeted cell as well as adjacent cells (bystander effect) in large tumor deposits. Pretreatment with unlabeled antibody is necessary to saturate normal cell populations and a dose of ¹¹¹In-labeled antibody is given to determine dosimetry. Eighty percent of relapsed low-grade B-cell lymphoma patients and 40% of relapsed intermediate-grade B-cell lymphoma patients respond to ⁹⁰Y-ibritumomab tiuxetan with median duration of remissions of 1 year (52). The effect is greater than with rituximab alone and occurs in patients previously treated with rituximab (53). Toxicities are related to myelosuppression from bone marrow injury, and careful marrow examination and ¹¹¹In-labeled dosimetry is mandatory pretreatment (54).

¹³¹I-tositumomab is a radioiodinated murine anti-CD20 monoclonal antibody (55). Radioiodine is attached to tyrosine residues on the antibody. The radionuclide emits both β particles and

γ rays. The β particles have lower energy and a shorter path length (1 mm) than those released by ⁹⁰Y. Further, the gamma emissions allow imaging and dosimetry, but create a health hazard necessitating patient isolation. Patients receive unlabeled tositumomab plus labeled compound, and images are obtained for dosimetry calculation. Then, 1 week later, a second dose of unlabeled and labeled tositumomab are given sufficient to yield the desired total body irradiation absorbed dose. Again, 80% response rate in relapsed follicular lymphoma patients and 40% response in relapsed transformed follicular lymphoma were achieved with 1-year median response duration (56). Preliminary studies of ¹³¹I-tositumomab therapy of previously untreated follicular lymphoma patients suggest excellent response rates (90%) with response durations in excess of 5 years (57,58). Toxicities included myelosuppression and hypothyroidism (due to radioiodine uptake by normal thyroid cells; 59).

Investigational Antibody Compounds

A large number of antibody-based drugs have entered cancer clinical trials in the last decade. Some of these agents have shown dramatic efficacy and safety and are continuing clinical testing (Table 55-2). Others will likely show promising activity within the next few years. For brevity and accuracy, we will focus on some of the drugs with promise for approval and clinical application.

Denosumab is a fully human monoclonal antibody to the receptor activator of nuclear factor- κ B ligand (RANKL; 60). Single subcutaneous doses have greater bone antiresorptive effect than pamidronate in patients with breast cancer and myeloma. Comparative studies with bisphosphonates in breast cancer are under way.

Nimotuzumab is a humanized anti-EGFR antibody that has been administered intravenously to children and adolescents with high-grade gliomas and resulted in a 20% response rate and 30% stable disease rate and no toxicities (84,85). Patients with pontine gliomas responded best.

¹³¹I-labeled murine anti-tenascin monoclonal antibody was injected directly into the surgically created resection cavity in patients with recurrent malignant gliomas (61). Patients then received chemotherapy. The median survival appears increased relative to historical controls treated with surgery and ¹²⁵I-brachytherapy. Toxicity includes myelosuppression and neurotoxicity.

¹³¹I-labeled murine anti-CD45 monoclonal antibody was combined with busulfan and cyclophosphamide for allogeneic stem cell transplant conditioning in patients with acute myeloid leukemia (62). Survival at 3 years was 1.5 times higher than historical controls conditioned with busulfan and cyclophosphamide alone.

Ticilimumab (CP-675,206) and ipilimumab (MDX-010) are human monoclonal antibodies to cytotoxic T-lymphocyte-associated antigen 4 (CTLA4). They block peripheral immunologic tolerance and trigger antitumor efficacy in melanoma patients (63,64,76). Among patients with metastatic melanoma, the complete and partial response rate was 13% and an additional 27% of patients had several years without disease progression. Toxicities were significant with autoimmune colitis, dermatitis, vitiligo, hypophysitis, and thyroiditis. Among hormone-refractory prostate cancer patients, ipilimumab had a 12% partial response rate (76).

Table 55-2 New Clinically Active Anticancer Antibodies

Antibody	Type	Antigen	Disease	Toxicity
Denosumab	Hu	RANKL	Breast cancer/myeloma	—
¹³¹ I-81C6	M/R	Tenascin	Glioma	Neurotoxicity
¹³¹ I-BC8	M/R	CD45	AML	—
Ticilimumab	Hu	CTLA4	Melanoma	Autoimmune disorders
Ipilimumab	Hu	CTLA4	Melanoma	Autoimmune disorders
⁹⁰ Y-epratuzumab	H/R	CD22	B-cell lymphoma	Myelosuppression
Galiximab	C	CD80	B-cell lymphoma	Fatigue
Epratuzumab	H	CD22	B-cell lymphoma	Infusion reaction
Pertuzumab	H	HER2	Breast cancer	Diarrhea
Catumaxomab	B	EpCAM/CD3	Cavity carcinomatosis	—
Oregovomab	M	CA125	Ovarian cancer	—
IMC-11F8	Hu	EGFR	Rectal cancer	Rash
CP-870,893	Hu	CD40	Melanoma	Thrombosis
¹⁷⁷ Lu or ⁹⁰ Y-J591	M/R	PSMA	Prostate cancer	Myelosuppression
hMN14-734/ ¹³¹ I-DTPA	B/R	CEA	Medullary thyroid cancer	Myelosuppression
BL22	M/T	CD22	Hairy cell leukemia	VLS
Nimotuzumab	H	EGFR	Pontine glioma	—

AML, acute myelogenous leukemia; B, bispecific; C, chimeric; H, humanized; Hu, human; M, murine; R, radionuclide conjugate; T, toxin conjugate; VLS,

Drug-related toxicities were also autoimmune including adrenal insufficiency and colitis.

⁹⁰Y-labeled 1,4,7,10-tetra-azacyclodecane-*N,N',N'',N'''*-tetraacetic acid-conjugated anti-CD22 humanized monoclonal antibody epratuzumab was administered to patients with both indolent and aggressive B-cell lymphomas (65). Treatment consisted of ⁹⁰Y-labeled epratuzumab with unconjugated epratuzumab weekly for two to four infusions and with ¹¹¹In-labeled epratuzumab on the first infusion for imaging and dosimetry. Patients with CD22⁺ disease had an 80% response rate with responses lasting years. Toxicity was myelosuppression.

Galiximab is a chimeric anti-CD80 monoclonal antibody targeted to follicular lymphomas (66). Four weekly intravenous infusions of up to 500 mg/m² yielded a response rate of 11% and a stable disease rate of 34% among relapsed or refractory follicular lymphoma patients. Toxicities were low grade, consisting of fatigue, nausea, and headache.

Epratuzumab is a humanized monoclonal antibody to CD22 antigen, which is present on normal and malignant B cells (67). Epratuzumab at 360 mg/m² plus rituximab at 375 mg/m² for 4 weeks yielded remissions in 70% of relapsed follicular and large cell lymphomas with remission durations of 1.5 years. This exceeds the historical results with rituximab alone, particularly for relapsed/refractory large-cell lymphoma. Toxicities were mild and consisted primarily of infusion reactions.

Pertuzumab is a humanized monoclonal antibody reactive with domain II of the extracellular domain of HER2 (68). The

antibody has a different target on HER2 than trastuzumab and inhibits HER2 dimerization. In a phase 1 trial, an overall 5% single-agent response rate was seen with remission in a pancreatic islet cell carcinoma and ovarian carcinoma (69). Toxicities were mild diarrhea, rash, and asymptomatic drops in cardiac ejection fraction.

Catumaxomab is a trifunctional, bivalent murine antibody reactive with EpCAM and CD3 and, via the Fc region, Fc-γ receptors 1 and 3 on accessory cells (monocytes and dendritic cells). The antibody stimulates an immune response at the tumor site. The agent was administered intraperitoneally to patients with peritoneal carcinomatosis (74). Treated patients had a median survival of 12 months versus 10 months in matched control patients receiving conventional therapy. There were no reported toxicities. Catumaxomab has also been given intrapleurally for EpCAM-positive malignant pleural effusions (80). There was a 40% response rate. Antirodent antibody responses were noted.

Oregovomab is a murine monoclonal antibody for CA125 antigen given intravenously monthly for three doses then every 3 months in totally resected ovarian cancer patients (77). Survival was 56% in treated patients versus 32% in controls. No toxicity was reported.

IMC-11F8 is a fully human anti-EGFR antibody that has shown a 5% remission rate already in a phase 1 dose-escalation study (81). Responders had rectal cancer and melanoma. Side effects are rash, nausea, vomiting, fatigue, and headaches.

CP-870,893 is a fully human anti-CD40 monoclonal antibody that activates costimulatory and MHC molecules on tumor cells stimulating immune responses (79). At 0.2-mg/kg doses, melanoma patients showed a 27% partial remission rate. Toxicities were cytokine release syndrome, transaminasemia, elevated D-dimer, venous thromboembolism, and headache.

J591, a mouse monoclonal antibody to prostate-specific membrane antigen, was deimmunized by specific deletion of human B- and T-cell-recognized epitopes and then conjugated to a macrocyclic chelating agent, DOTA. The J591-DOTA was then labeled with ¹⁷⁷Lutetium or ⁹⁰Yttrium (70,71). Lutetium is similar to yttrium but has a longer half-life, lower energy, and shorter path length (1 mm). Labeled antibody was administered up to three doses to metastatic prostate cancer patients. Biologic activity was seen in 10% of patients with both agents, and toxicity was myelosuppression.

BsMab hMN14-734 is a humanized anti-CEA/anti-DTPA-indium monoclonal antibody. Five days after infusion, patients receive ¹³¹I-labeled bivalent hapten DTPA. Among patients with high-risk or bone marrow-positive metastatic medullary thyroid carcinoma, radioimmunotherapy doubled overall survival to 110 months versus 61 months (72). Toxicity was myelosuppression.

BL22 is a disulfide-stabilized murine sFv anti-CD22 fused to *Pseudomonas* exotoxin fragment PE38 (82). BL22 given at doses of 30 µg/kg intravenously every other day for three doses to purine analog-resistant hairy cell leukemia patients yielded an 80% remission rate. Toxicities were hypoalbuminemia, fatigue, transaminasemia, and edema.

Discussion

Monoclonal antibodies have become an important part of the anti-cancer armamentarium. There are eight approved agents in clinical practice and several more likely to obtain FDA approval in the near future. Problems with production have been mostly overcome with advances in biotechnology.

Because all the antibody reagents were produced with recombinant DNA methods, these compounds offer the opportunity for further improvements in design to reduce immunogenicity, enhance affinity and stability and half-life and effect function and tissue penetration and further improve ease of production. Genetic engineering techniques for these manipulations have become routine

and, with structural information, a set of rules have begun to be formulated (83). Thus, pharmacologic limitations in clinical trials can be addressed without abandoning targets or disease states.

The mechanism of antitumor efficacy varies greatly among agents. Some show signal blockade, some act via immune effector mechanisms, and some conjugates kill by delivery of radionuclides, cytotoxic drugs or peptide toxins. There does not appear to be a preference for one approach versus another, though naked antibodies generally show less myelosuppression or vascular injury.

Which patients are most likely to respond? Patients with minimal tumor burden appear most likely to achieve remissions with these biologic agents. Biomarkers to predict response may also be useful: FISH for trastuzumab and flow cytometry or immunohistochemistry for rituximab, alemtuzumab and gemtuzumab ozogamicin. Since these agents target particular antigens or pathways, determination of the surface expression of the antigen and the tumor dependence on the relevant pathway may be useful.

Combinations of antibodies or antibody plus cytotoxic drugs or radiotherapy appear to yield at least additive if not synergistic benefit. Thus, after initial phase 1/2 trials, most agents are used clinically in combinations. The nonoverlapping toxicities facilitate such an approach.

Interestingly, antibodies do produce significant toxicities which vary for each agent. Although most antibodies produce a mild to moderate infusion reaction with fever, chills, hypoxemia, and hypotension, more severe toxicities are antigen-specific (e.g., trastuzumab and cardiac failure, alemtuzumab and immunocompromised host infections, gemtuzumab ozogamicin and liver failure).

Humoral immune response has been decreased but persists as a problem. Antibodies with human constant regions (chimerics), human variable domain frameworks and constant regions (humanized), and altered murine variable domain residues (deimmunized) have low but some immunogenicity. Even human antibodies have some idiosyncratic immunogenicity (83).

The high cost of the drugs will likely have an impact on their widespread use in malignancies (78). With the limited economic resources available for patients and the increasing demands on the health care system, future work to reduce the costs of these agents will be as important as increasing their numbers and applications.

Nevertheless, this is an exciting time in medical oncology, and careful tailoring of therapy to the biology and psychology of the patient should enhance the duration and quality of cancer patient life.

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Gene Therapy and Oncolytic Viruses

Genetic alterations are the driving force behind cancer development and progression. It follows that cancer could potentially be treated by correcting these alterations using gene therapy or by agents that kill cells by mechanisms based on these genetic alterations. Approaches of these kinds have a number of potential advantages. Vectors and viruses can be engineered in countless ways to achieve specificity and potency, and can be designed and tested using tools that are routine in contemporary molecular biology laboratories. For these reasons, a dazzling array of creative concepts has been described over the last 20 years. However, few of these have been tested extensively in the clinic, and none has yet completed a successful phase 3 clinical trial in the United States or Europe. Therefore, gene therapy and oncolytic viral therapy have not yet lived up to their promise and have not yet entered the mainstream of medical oncology, by any means. In this chapter, we discuss the potential and challenges of these novel technologies. We focus on the main strategies that have been evaluated clinically. These include the design of agents that kill cancer cells by gene replacement or by disruption of oncogenic signaling pathways. For example, therapeutic agents have been developed that reintroduce the wild-type *p53* tumor-suppressor gene or destroy RNA encoding the oncogene *K-ras*. The delivery of toxic genes and genes that convert prodrugs into toxic metabolites has been tested clinically extensively and has produced exciting results. Furthermore, we highlight strategies that aim at modulating the immune-response in order to achieve anticancer effects. Finally, we discuss the basic and clinical aspects of the use of replication-competent viruses, either in their natural configuration or genetically modified, to selectively kill cancer.

Historically, gene and viral therapies are coming full circle. Some of the first gene therapy experiments were conducted by Dr. Rosenberg at the NCI in the late 1980s and aimed at generating T-cell populations that would specifically target cancer cells (1). Twenty years of intense research later, his team was able to report complete remissions in two patients with melanoma who were treated with a more sophisticated development of this approach (2). Similarly, the use of viruses to kill cancer cells has been reported as early as 1956 (3). In 2003, the worldwide first approval for a gene therapy agent was granted by the Chinese regulatory authorities to Gendicine, a replication-incompetent adenovirus expressing the tumor-suppressor protein *p53*. This was followed

by H101, a genetically modified adenovirus designed to replicate in and kill cells with altered *p53* pathway, which was approved in China in 2005 for the use in patients suffering from head and neck cancer (4).

Killing Cancer Cells by Gene Replacement and Gene Knock-Out

Cancers develop through loss of function of key regulatory genes known as tumor suppressors as well as through activation of proto-oncogenes. Loss of *p14*, *p16*, *p53*, or *PTEN* occurs at high frequencies in most tumor types. Loss of antigen-presenting cells (APCs) occurs in the majority of colorectal cancers, loss of *RB* is frequent in small cell lung cancers, amongst many other examples (5,6). Mutations or deletions inactivate products of these genes, or their expression is suppressed by hypermethylation. Clonal evolution of tumors depends on loss of these functions: Replacement of functional versions of these genes should therefore reverse this process. Indeed, when tumor suppressors are reexpressed in tumor cells by gene delivery, tumor cells die or growth arrest. This has been shown for *APC*, *p16*, *p21*, *p27*, *RB*, *p14ARF*, *p53*, *PTEN*, *APC*, and *BRCA1*, *BRCA2*, among others (4).

In contrast, normal cells receiving additional copies of these genes appear to be unaffected, at least in the cases that have been tested so far. This has been documented most clearly for *p53*: delivery of this tumor suppressor to normal bronchial epithelial cells had no effect on cell growth, suggesting a therapeutic window of more than two orders of magnitude (7). This may be because tumor cells have additional defects that make them more sensitive to the effects of reexpressing *p53*. For example, almost all tumor cells have a defect in the *RB* checkpoint and therefore are less able to undergo growth arrest. Normal cells, in contrast, undergo G_1 arrest when *p53* is active, and this can protect them from apoptosis. In addition, the negative regulator of *p53*, *Mdm2*, is itself a *p53* effector (8). In tumor cells lacking *p53*, *mdm2* levels are often low. When *p53* is reexpressed in these cells, it is not susceptible to *Mdm2*-mediated degradation, and is therefore more active. In normal cells, *Mdm2* constantly degrades *p53* and maintains low *p53* activity. In addition, *p53* promotes a bystander effect on

uninfected tumor cells, possibly through anti-angiogenesis (9), secretion of soluble pro-apoptotic proteins (10), and immune up-regulation (11,12). In agreement with these theoretical considerations, clinical safety and some evidence for antitumor activity of an adenoviral vector expressing wild-type *p53* under the control of a Rous-Sarcoma-Virus promoter (Gendicine) have been demonstrated and led to approval of this agent in China for the use in head and neck cancer (13). At this point, 2,500 patients have been treated with this virus. Self-limiting fever and flulike symptoms were the main adverse events; no severe side effects occurred (13). Long-lasting responses with this agent were observed in studies in patients with head and neck cancer. Very high response rates, including 64% complete responses were observed following intra-tumoral injection of Gendicine in combination with radiation therapy (14,15). A similar virus that delivers wild-type *p53* under the control of a constitutively active cytomegalovirus (CMV) promoter (AdCMV-*p53*; Advexin) is in a phase 3 clinical trial in the United States in patients with cancer of the head and neck (16). The results from these trials will be decisive for the future of this approach in the Western Hemisphere.

Activation of proto-oncogenes such as *Ras*, *B-Raf*, *Myc*, or *EGFR*, through various mechanisms is a key factor in carcinogenesis. For example, mutations of the *Ras* oncogene occur in many types, including pancreatic cancer, where about 90% of the cases carry a mutation of the *K-ras* gene (17). Knock-out of oncogenes driving tumor development in transgenic mouse models of cancer can lead to complete regression, confirming their potential value as drug targets (18). Antisense technology has been studied as a strategy to knock-down expression of oncogenes. This approach is based on the possibility of inhibiting transcription of a particular mRNA by transfecting short, double-stranded DNA oligonucleotides into target cells that bind and inactivate the target mRNA in a sequence-specific manner (19). The development of oligonucleotides with modified DNA backbone that increases the stability of the molecule in vivo allowed for the development of clinical protocols involving intravenous application of antisense molecules targeting for example mutant *K-ras* or the anti-apoptotic genes *BCL-2* and *survivin* (20). Clinical trials with oblimersen sodium (G3139), an antisense oligonucleotide directed against *BCL-2*, have demonstrated increased survival of patients with advanced melanoma in combination with chemotherapy compared to chemotherapy alone (21). An interesting variant of the antisense approach which is currently tested clinically represents GL163L, a lipid-modified 13-mer DNA-oligonucleotide that acts as a telomerase RNA template antagonist (22).

The discovery of the possibility of silencing gene expression in mammalian cells with high efficiency using RNA interference (RNAi) has spurred renewed interest in the gene knock-down as a therapeutic strategy. This approach, first described in the worm *C. elegans*, takes advantage of a cellular gene silencing machinery that involves the RNA-induced silencing complex (RISC) and degrades double-stranded RNAs with high efficacy (23). The introduction of short RNAs with complementary sequence to any cellular transcript activates this mechanism. RNAi can silence target genes with high efficacy. However, off-target effects which result in sequence-specific though unpredictable knock-down of

additional genes represent a potential problem that has not been fully resolved at this point (24). Nevertheless, novel targeting and delivery mechanisms have been developed that hold the promise of therapeutic application of RNAi-based agents in cancer (23).

Ribozymes represent a separate class of RNA-based agents. In this case, "hammerhead" shaped RNAs catalyze the sequence-specific degradation of target RNA. Successful knock-down of targets such as *K-ras* and *hTERT* using in vitro models has been described (25,26). While this strategy has worked well in pre-clinical models and clinical trials have demonstrated safety of this approach (27), the antitumor efficacy of such agents has been disappointing and the approach is currently not being further pursued clinically.

Killing Cancer Cells by Delivering Toxic Genes

Viral vectors have been used to deliver a variety of toxic genes to tumor cells, taking advantage of altered properties of tumor cells to kill them selectively. Early studies attempted to take advantage of tumor cell proliferation relative to surrounding normal cells to kill brain tumors selectively. Retrovirus vectors were used to deliver the *herpes simplex virus thymidine kinase* gene, which converts the pro-drug ganciclovir to a toxic product. Since retroviruses only integrate in proliferating cells, gene delivery and expression would be tumor selective in the context of normal, nonproliferating brain cells (28). A further advantage of this approach was thought to result from the "bystander effect," the killing of uninfected neighboring cells that occurs when HSV-expressing cells are exposed to ganciclovir, which can be observed in vitro and in vivo (29). To increase transduction of target cells with retroviruses, virus producing cells (VPCs) were used to inoculate target tumors instead of virus suspension. Preclinical studies in a rat glioma model demonstrated that this delivery of a retrovirus expressing HSV-TK resulted in high transduction levels and frequent tumor regressions following ganciclovir administration (30). Initial clinical studies were promising and demonstrated in responses of small glioblastomas following VPC injection (31). However, when standard therapy (surgical resection and radiotherapy) was compared with standard therapy plus injection of retrovirus-producing cells in a phase 3 trial, no differences in progression-free and overall survival were observed between both groups (32). Smaller, preoperative, studies suggested that lack of transduction of tumor cells is the dominant reason for the failure of this approach (33). Despite the disappointing results of this trial, it represents an early and innovative effort to use gene therapy to kill cancer cells selectively.

In contrast to retroviral delivery, a randomized study of adjuvant injection of a nonreplicating adenovirus construct expressing HSV-TK into the tumor bed following resection of glioblastoma multiforme resulted in an improved median survival from 37.7 weeks in the control group to 62.4 weeks in the treatment group (34). CTL102 is a nonreplicating adenovirus expressing a bacterial nitroreductase enzyme, which converts the weak monofunctional alkylating agent CB1954 into a highly cytotoxic bifunctional alkylating agent effective in replicating and quiescent

cells (35). Injection of this virus into locally relapsed prostate cancer and infusion of the prodrug was well tolerated in early clinical trials and preliminary evidence for antitumor activity was observed, mainly in form of long-lasting stabilization of serum prostate-specific antigen (PSA) levels (36). The efficacy of treatments utilizing nonreplicating viruses is particularly dependent on transduction of a large number of tumor cells. A preoperative trial of intratumoral injection of this virus in patients with respectable primary or secondary liver malignancies was conducted in order to address this question. This strategy allows analysis of the entire resected tumor. Interestingly, dose-dependent transduction of tumor cells was demonstrated. After injection with single dose of 1×10^{11} viral particles, up to 50% of tumor cells demonstrated expression of the transgene (37). Encouraging results were also obtained in a small series of patients with primary or secondary liver cancer who received intratumoral injections of CTL102. Safe administration of this virus through the intravenous route was also demonstrated (38).

Many other strategies have been devised to express prodrug-converting enzymes, or other potentially toxic genes, in cancer cells selectively. Most of these depend on tumor-selective gene expression to drive the gene of interest, such as the promoters for the androgen receptor or PSA (selective for prostate cancer; 39–41), for tyrosinase (selective for melanoma; 42), or for α -lactalbumin (selective for breast cancer; 43). More generic tumor-selective-specific promoters include E2F-1, which is up-regulated through loss of the RB checkpoint, and telomerase reverse transcriptase (TERT), which is also up-regulated in most cancers (44,45).

Genes That Boost the Immune System

The promise of harnessing the immune system to attack cancer cells has not been fulfilled to the extent of initial expectations. One approach to boosting antitumor immune responses involves administration of cytokines, to increase the activity of immune effector cells systemically, or to enhance presentation of antigens in tumor cells themselves. A wide range of cytokine genes have been engineered into an equally wide range of vectors and viruses. IL-2, IL-4, and IL-12 have been studied extensively, as well as interferons, members of the TNF family and GM-CSF. TNFerade® is an exciting example of this approach. This replication-incompetent adenovirus expresses TNF under the control of the early growth response factor 1 (*egr-1*) promoter, which is strongly activated in response to cellular stress (e.g., ionizing radiation or chemotherapy; 46). TNFerade was injected in phase 1 studies to up to 4×10^{11} pu (particle units) and no dose-limiting toxicities occurred. In agreement with this favorable safety profile, serum TNF levels remained consistently low. However, in esophageal cancer, relatively high rates of thromboembolic complications occurred, which were potentially induced by the study medication. This problem was not observed in trials of this agent in pancreatic cancer. In this disease the maximum tolerated dose (MTD) of TNFerade in combination with chemoradiation treatment was determined to be 4×10^{11} pu. At that dose level, clinical responses and evidence for prolonged median survival was seen in an interim analysis of a phase 3 study in

locally advanced pancreatic cancer in combination with chemoradiotherapy (47,48).

Naturally Occurring Virus That Replicate in Cancer Cells Selectively

Cancer cells provide an environment that is permissive for replication of a number of naturally occurring viruses. This is because checkpoints and defense mechanisms are disabled in cancer cells, allowing them to grow and survive and to evade detection by the immune system. In some cases these mechanisms are also used to defend normal cells against virus replication. Cancer cells may therefore be vulnerable to virus infection while normal cells are protected. Since infection usually leads to cell death, this vulnerability could potentially be exploited for cancer therapy. Indeed, several naturally occurring viruses are under clinical evaluation in a variety of cancer indications (Table 56-1).

Reoviruses, for example, replicate selectively in many cancer cells. During infection of normal cells, their double-stranded RNA genomes activate a cellular protein kinase (PKR) that restricts viral replication by blocking translation of viral mRNA. For reasons that are unclear, this kinase activity is suppressed in cancer cells in which the Ras pathway is hyperactivated, allowing productive viral replication (49). Based on this selectivity, and the capacity of reoviruses to replicate quickly and kill infected cells, reoviruses are undergoing clinical evaluation. One of these, REOLYSIN®, is being tested in Canada, the United Kingdom, and the United States using clinical protocols that include local or systemic delivery of REOLYSIN® as a monotherapy, and local delivery in combination with radiation therapy for patients with advanced cancers. Intravenous administration of this virus was well tolerated in a phase 1 study. While no objective tumor responses were observed, disease stabilization of up to 6 months was observed in a subset of patients (50).

The rhabdovirus vesicular stomatitis virus (VSV) has a single-stranded RNA genome. Selectivity for cancer cells is thought to be the result of their failure to illicit a protective interferon response, thus allowing lytic replication. VSV variants with mutations in the matrix (M) protein enhance VSV's effectiveness, at least in animal models (51). Systemic delivery of VSV has been shown to be effective and safe against laboratory models of multifocal and invasive malignant gliomas (52). M protein mutant viruses have also shown efficacy against prostate cancer cell lines and others (51).

Measles viruses, like VSV, contain negative-stranded RNA genomes, but are members of the paramyxovirus family. Replication-competent attenuated Edmonston B measles vaccine strain (MV-Edm) is nonpathogenic and has potent antitumor activity against several human tumors. The virus is selectively oncolytic, eliciting extensive cell-to-cell fusion and ultimately leading to cell death. An attenuated strain of measles virus has been genetically engineered to produce carcinoembryonic antigen which can be used as a serum marker of virus replication (15). This virus had potent antitumor activity against gliomas in vitro and in animal models (53). This virus is undergoing clinical evaluation in patients with glioblastoma multiforme and multiple myeloma.

Table 56-1 Cancer-Selective Viruses in Clinical Trials

Naturally Occurring Viruses	Genome	Basis of Selectivity	Ref.
Reovirus	dsRNA	Ras suppresses PKR	48
VSV	ssRNA (–)	Tumor cells fail to respond to IFN	50
Measles	ssRNA (–)	Not clear	52
NDV	ssRNA (–)	Selective induction of apoptosis	54
Engineered viruses			
HSV G207	DNA	Viral ribonucleotide reductase (–) Neurovirulence genes (–)	59
HSV1716	DNA	As above	61
NV1020	DNA	As above	62
Vaccinia	DNA	Attenuated in normal cells	65
ONYX-015 (Ad dl1520)	DNA	E1B 55K (–)	68

DS, double strand; ss, single strand.

Lytic strains of the avian paramyxovirus Newcastle disease virus (NDV) selectively kill cancer cells in culture and in mouse models (54). The molecular basis of selectivity is not fully understood, but appears to be facilitated by high levels of N-myc, at least in neuroblastoma cells. Cytotoxicity is due to multiple caspase-dependent pathways of apoptosis independent of interferon signaling competence (54,55). Poliovirus and West Niles Virus are also RNA viruses with tumor cell selectivity, in these cases with a positive-strand genome and are being evaluated for cancer therapy. Several phase 1 studies of intravenously infused NDV have been performed using various doses and administration schedules (56,57). Main toxicities included moderate flu-like symptoms and mild gastrointestinal symptoms. Interestingly, a two-step intrapatient dose-escalation of the NDV strain PV701 aiming at desensitizing resulted in significant reduction of the intensity of adverse events. Patients developed only moderate levels of neutralizing antibodies and the serum clearing of virus was not significantly different during the course of treatment (58). Disease stabilization as well as objective tumor responses were observed in phase 1 studies, in particular in patients who had received higher doses. A complete remission was observed in a patient with glioblastoma multiforme treated with the NDV strain NDV-HUJ (56).

Viruses Engineered to Replicate Selectively

In addition to naturally occurring viruses, many efforts have been made to engineer viruses to replicate in tumor cells selectively. Such agents kill cells through lytic mechanisms and potentially spread from one infected cell to another, amplifying the dose of the selective killing agent. Selectivity for cancer cells can be achieved by several strategies. The first uses DNA synthetic enzymes produced

by proliferating tumor cells to support replication of DNA viruses that are otherwise defective. The second takes advantage of genetic defects in cancer cells that supply functions that have been specifically deleted from the oncolytic agent, and third uses tumor-selective promoters to drive replication of conditionally replicating viruses.

One of the first viruses designed to replicate in cancer selectively were herpes simplex viruses (HSVs) that had been engineered so that they were unable to express viral genes necessary for DNA replication, such as thymidine kinase or ribonucleotide reductase. Proliferating cells would provide these essential functions, whereas resting normal cells would not. HSV G207 is an example of such an oncolytic virus. In addition to inactivation of a subunit of the viral ribonucleotide reductase gene, both copies of the neurovirulence gene, γ 34.5 gene are deleted to further reduce replication in normal tissues (59). Although direct tumor cell killing represents a major mechanism of action of these viruses, evidence from experiments in immunocompetent mouse models suggests that also a vaccination effect, mediated by activated T-lymphocytes contributes to the effect (60). Phase 1 clinical trials of G207 and a related virus, HSV1716, have been completed and demonstrated safety of these viruses (61). Another related HSV mutant, NV1020, has been tested in a phase 1 study in patients with hepatic metastases from colorectal cancer. The virus was administered into the hepatic artery in a phase 1 study. Only mild toxicity was observed and a decline in carcinoembryonic antigen (CEA) levels was suggestive of some antitumor activity (62). Many innovative approaches have been employed to improve the clinical value of HSV viruses, including expression of prodrug-converting enzymes to elicit bystander cells and the addition of genes encoding cytokines, to boost immune recognition of tumor cells. The latter approach has been taken into the clinic in form of the HSV mutant OncoVex^{GM-CSF} a conditionally replicating

HSV-1 mutant that expresses the cytokine GM-CSF. A phase I trial with intratumorally injected Oncovex GM-CSF demonstrated that this agent is well tolerated (63). Side effects included mainly fever, flulike symptoms, and inflammation at the injection site. Clinical evidence for tumor necrosis was found, however, no detailed objective response assessments have been published at this point.

Using a similar strategy, attenuated strains of another large DNA virus, Vaccinia, have been created and shown to replicate selectively in cancer cells. For example, Bartlett and coworkers showed that Vaccinia mutants deleted for the thymidine kinase and Vaccinia growth factor genes are severely attenuated in normal cells but grow efficiently in cancer cells, presumably because the deleted functions are provided by the permissive cancer cell environment (64). Selectivity has been increased further by deleting Vaccinia genes that encode serine protease inhibitors and thus suppress apoptosis (65). Clinical studies with tumor-selective Vaccinia viruses are under way.

While G207 and related viruses were engineered to take advantage of permissive conditions in cancer cells relative to quiescent cells, ONYX-015 was designed to exploit lack of functional p53 in tumor cells. ONYX-015 is an adenovirus that lacks the *E1B 55K* gene. This gene encodes a protein that binds p53 and targets it for degradation. In the absence of *E1B 55K*, ONYX-015 was expected to replicate poorly in normal cells, in which functional p53 could abort lytic, productive replication. In contrast, cancer cells should be permissive for ONYX-015 since *E1B 55K* should be unnecessary in cells that lack p53 (66).

Extensive analysis of the molecular mechanisms underlying ONYX-015 replication revealed that the virus does indeed replicate selectively in tumor cells. While replication of this virus is mediated through the p53 pathway, tumor selectivity is mostly based on the ability of tumor cells to complement other functions of *E1B 55K* unrelated to p53 (67–69). These functions relate to the ability of *E1B 55K* to facilitate export of viral mRNAs and shut down host protein synthesis. Nonetheless, ONYX-015 advanced to phase 3 trials in the United States after encouraging signs of efficacy and safety and efficacy (Table 56-2). The analysis of clinical tumor specimens obtained following injection of ONYX-015 provided unambiguous evidence for its tumor-selectivity. Replicating virus was found (1) in head and neck cancer following intratumoral injection (Figure 56-1); (2) liver metastases from gastrointestinal cancer after intra-arterial injection; and (3) lung tumors following intravenous injection (70–72). Furthermore, objective tumor responses were observed in several clinical phase 2 studies. Single-agent treatment with ONYX-015 induced objective responses in 14% of patients with recurrent head and neck cancer (73). In a subsequent study, the virus was combined with standard chemotherapy. Only a single tumor was injected with ONYX-015, leaving a subgroup of patients with additional uninjected control lesions. Interestingly, the response rate of tumors injected with the virus was significantly greater than those of noninjected lesions and the time to progression was significantly longer for injected tumors (70). Further evidence of antitumor activity of ONYX-015 was found in three patients with 5-FU/leucovorin-refractory liver metastases from colorectal cancer that experienced minor responses (30%–48%

shrinkage) following intra-arterial infusion of ONYX-015 into the hepatic artery (74).

Meanwhile, a closely related adenovirus, H101, was approved for treatment of head and neck cancer in China (4) after a clinical phase 3 study demonstrated a dramatically higher tumor response rate in patients who had received H101 in combination with cisplatin and 5-fluorouracil (78.8% vs. 39.6%, respectively; 75).

Delta-24 (also known as *dl922–947* or ONYX-838) is another adenovirus mutant that targets cancer cells selectively (76). The *E1a* region contains a small deletion that prevents binding to RB. As a result, this virus cannot replicate efficiently in normal cells, since RB represses E2F activity that is essential for replication (in addition to transcribing genes involved in DNA synthesis, E2F activates transcription of the viral *E2* region, which is how E2F was first named and identified). In cancer cells, E2F activity is not repressed by RB, because RB itself is mutated or inactivated indirectly through loss of *p16INK4a*, amplification of cyclin D1, or by other means. Tumor cells therefore provide a permissive environment for replication of Delta-24. A modified version of Delta-24 is expected to enter clinical trials in 2007. This virus, Delta-24 RGD, has been engineered to increase infectivity by addition of an RGD sequence in its fiber gene (see following sections; 77). Likewise, adenoviruses designated KD1 and KD3 contain two small deletions in *E1A* that abolish its binding to pRB but leave the ability of *E1A* to transactivate viral genes intact. These have been shown to replicate with great efficiency in tumor cells, but fail to replicate efficiently in normal cells (78).

A second-generation version of Delta-24 was engineered to express human p53 (79,80). Ad24-p53 more effectively killed most human cancer cell lines tested in vitro than did its parent Ad24 and had significant activity against xenografts in vivo (79). To further improve potency of this virus, the p53 transgene was engineered so that it is resistant to degradation by Mdm2 (79). More recently, adenoviruses have been engineered to take advantage of unregulated TCF transcriptional activity, a characteristic of colorectal cancer cells that lack the APC tumor suppressor, or contain activating mutations in β -catenin (81,82).

An array of creative approaches has been used to make viruses replicate selectively based on abnormal transcription activity in cancer cells. These include viruses that use unregulated E2F activity resulting from loss of the RB tumor suppressor pathway to drive the E2F-1 promoter, the telomerase promoter, prostate-specific promoters, and regulatory elements to drive proliferation in prostate cancer cells, and many others.

Challenges and Future Perspective

Preclinical Development

Gene therapeutic agents and oncolytic viruses have highly diverse physical and biologic properties and act through complex molecular mechanisms, making predictions about their pharmacologic and pharmacodynamic behavior in humans difficult. Preclinical model systems are therefore particularly important elements in the selection process for further clinical development.

Table 56-2 Clinical Studies of ONYX-015

Author (ref.)	Phase	Cancer Type	n	Route ^a	Chemotherapy	Response (%) ^b
Chiocca ¹²⁷	I	Glioblastoma	24	IT	—	SD, 1 (4)
Ganly ¹⁰³	I	Head and neck (recurrent)	22	IT	—	PR ^d , 3 (14) MR ^d , 2 (9) SD ^d , 8 (36)
Nemunaitis ⁷³	II	Head and neck (recurrent)	40	IT	—	CR, 3 (8) PR, 2 (5)
Khuri ⁷⁰	II	Head and neck (recurrent)	37	IT	5-FU/cisplatin	CR, 8 (27) ^c PR, 11 (36)
Makower ¹²⁸	II	Hepatobiliary	20	IT	—	PR, 1 (6) SD, 1 (6)
Mulvihill ¹⁰⁴	I	Pancreas	23	IT	—	SD ^d , 16 (70)
Hecht ¹²⁹	II	Pancreas	18	IT	Gemcitabine	PR, 2 (11) MR, 1 (6)
Vasey ¹⁰⁷	I	Ovarian	16	IP	—	SD, 4 (25)
Reid ⁷¹	I	Gastrointestinal (liver metastases)	11	HAI	5-FU/leucovorin	PR, 1 (9) SD, 2 (18)
Reid ⁷⁴	II	Gastrointestinal (liver metastases)	27	HAI	5-FU/leucovorin	PR, 3 (11) MR, 4 (15) SD, 9 (33)
Nemunaitis ⁷²	I	Lung metastases	10	IV	Carboplatin/taxol	SD, 8 (80)
Nemunaitis ¹³⁰	I	Metastatic tumors	10	IV	CPT-11/5-FU or IL-2	SD, 4 (40)
Hamid ¹³¹	II	Colorectal	18	IV	—	SD, 8 (39)
Galanis ¹³²	I-II	Sarcoma	6	IV	—	PR, 1 (17) SD, 4 (67)

—, no chemotherapy given.

^a IT, intratumoral injection; HAI, hepatic artery infusion; IP, intraperitoneally.^b CR, complete response; PR, partial response; MR, minor response; SD, stable disease.^c Response rates of injected lesions.^d Response assessment of injected tumor only.

Mouse xenograft tumor models of human cancer cell lines in nude mice allow efficient assessment of antitumor activities of novel gene delivery and oncolytic agents in a broad variety of human tumor types. However, these models have significant limitations due to the lack of a functional immune system and differences in structure and composition of the tumor stroma. To address these limitations, immunocompetent tumor models have been developed. Such models have been instrumental for assessing the impact of immune-modulatory genes in the genome of oncolytic adenoviruses on virus replication and antitumor effect (83). In addition, orthotopic implantation of allografts in immunocompetent models allows testing novel vectors in tumors growing a organo-typic microenvironment that more closely resembles the situation in humans (84–86). Despite these improved murine models there are remaining limitations. For example, normal and malignant mouse tissues only poorly support replication of human adenoviruses. Another example is the difference between the sequences of human and mouse cytokines, which makes the generation of mouse-specific variants of immune-modulatory agents

necessary. Other species offer potentially advantageous features. For example, normal and malignant Syrian hamster cells support adenovirus replication. Using this model, intratumoral injection of an oncolytic adenovirus resulted not only in suppression of the primary tumor but also of distant metastases following virus entry into the bloodstream (87). Similar results were obtained in cotton rats (88). In addition, replication-competent viruses for the use in canine models have been developed (89).

Biodistribution and systemic effects of novel gene therapy and oncolytic viral agents are fundamentally different from small-molecule or antibody-based anticancer therapies and therefore difficult to predict. To address this, novel in vivo imaging strategies allowing for real-time monitoring of the effects of such agents in animals as well as in humans have been developed and will play a major role in the further development of this therapeutic approach. Distribution of nonviral and viral particles can be directly assessed by radioactive or fluorescent labeling. The biodistribution of liposomes, for example, can be followed after labeling with radioactive isotopes (e.g., ^{99m}Tc) or gadolinium by scintigraphy or magnetic

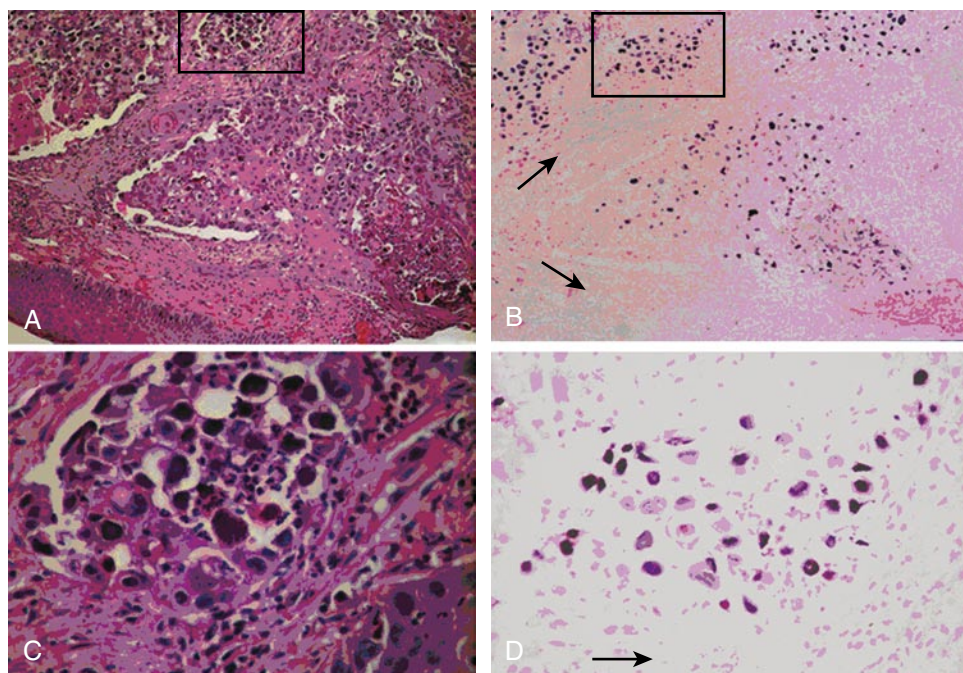


FIGURE 56-1 Demonstration of tumor-selective replication of ONYX-015. A tumor biopsy from a patient with cancer of the head and neck was obtained following intratumoral injection of ONYX-015. Hematoxylin and eosin staining (**A, C**) of tissue sections reveals tumor nests within normal stroma. In situ hybridization for adenoviral DNA was performed on parallel tissue sections and demonstrates evidence for viral replication exclusively in tumor cells (**B, D**). Black arrows, normal stroma; black box, tumor cell nest shown at high magnification in **B** and **D**.

resonance imaging (MRI), respectively (90,91). A variety of strategies has been pursued to monitor viral agents. Green-fluorescent protein and firefly luciferase are transgenes that allow detection of cells infected with viruses carrying an expression cassette for either of these genes through the detection of fluorescent light or bioluminescence, respectively (92,93). The use of prodrug-converting enzymes opens the possibility to use the enzyme activity for imaging purposes. The most developed approach is expression of the prodrug-converting enzyme HSV-Tk, which not only converts ganciclovir into cytotoxic phosphorylated derivatives but also phosphorylates uracil-derivates labeled with radioactive iodine and acyl-guanosines labeled with radioactive Fluor, which are then retained within the cell and detectable by positron emission tomography (PET). This approach has been successfully used in a variety of vectors in small animals. Recently, it has also been demonstrated to be an effective imaging strategy in a pilot study in patients with liver cancer (94). A similar approach involves the vector-mediated delivery of receptors with only limited physiologic expression (e.g., the dopamine D2 receptor or the somatostatin receptor subtype 2). PET imaging probes for both receptors are clinically available for imaging of neuroendocrine tumors. A third technology has been developed using the sodium-iodide-symporter, which is a transmembrane transport protein that is physiologically predominantly expressed in the thyroid gland (95). Ectopic expression of this protein leads to accumulation of radioactive iodine, which can be used for detected by radionuclide imaging using a gamma camera or PET. The potential advantage of this approach is that cytotoxic radionuclides such as ^{131}I can be used therapeutically (96).

The complexity of the host–vector interaction and the resulting dynamics of virus and tumor cell replication are theoretically

amenable to in silico modeling of virus and tumor cell population dynamics. Such mathematical models created important insights into the kinetics of HIV infection and treatment (97,98) and have been, at a theoretical level, developed for oncolytic virus and their interaction with tumor cells and the host immune system (99,100). Experimental validation of these models is being actively pursued by several laboratories.

Safety and Toxicity

The use of viruses either as vectors for the delivery of therapeutic genes or, in mutant form, as therapeutics themselves raised significant concerns not only in regards to the safety of individuals treated with such agents but also because of the potential risks for others. In particular, the occurrence of recombinant viruses that regain wild-type properties or demonstrate even greater toxicity was feared. For this reason, extensive safety studies were performed. For example, the biohazard potential of AdCMV-p53 was investigated in France in the context clinical trials of this virus in head and neck cancer. No evidence for any environmental risk of intratumoral injection of the virus was found (101).

The safety of administration of a replication-competent virus has been demonstrated comprehensively for ONYX-015. This virus has been tested in a variety of clinical settings (summarized in Table 56-2), which included its application as a mouthwash in patients with oral leucoplakia (102), intratumoral injection in a variety of cancer types (103–106), intraperitoneal injection in patients with ovarian carcinoma (107), infusion into the hepatic artery in patients with gastrointestinal tumors metastatic to the liver (74), and intravenous infusion in patients with lung tumors (72).

The leading toxicities included classical symptoms of an acute viral syndrome, such as flulike symptoms, fever, chills, and rigors, as well as pain at the injection site. Since the liver takes up viral particles efficiently and hepatotoxicity had been observed in animal models, it is important to note that most patients did not show evidence of hepatotoxicity, which is potentially attributable to the localization of the adenovirus receptor CAR at sites of cell–cell contact in hepatocytes (108). In patients who had been treated with doses of above 2×10^{12} viral particles intravenously, however, transient elevation of ALT approximately four times the upper normal limit was observed (72), suggesting a potential dose-dependent hepatotoxicity of ONYX-015. This adverse effect was only transient and not dose-limiting. Nevertheless, the potential of severe and possibly fatal adverse effects of gene therapy using viral vectors became evident when a patient with the nonmalignant, metabolic disease ornithine transcarbamylase deficiency was treated at the University of Pennsylvania with a nonreplicating adenovirus expressing this enzyme. After receiving the highest virus dose in that trial into the hepatic artery, the patient developed severe fever, evidence of disseminated intravascular coagulation, and died within 4 days from multi-organ failure (109). Such severe adverse events have not been observed with local or systemic administration of replication-competent viruses but clearly serve as warning examples that should guide the development of clinical gene therapy protocols.

Immune and Cytokine Response

A major concern with respect to the use of viral particles as therapeutic agents is the induction of neutralizing antibodies that could limit the efficacy of such agents. In the case of adenovirus this is of particular relevance as at least 50% of patients present with pre-existing antibodies against adenovirus type 5, resulting from earlier infections (71,73,74,103,110,111). It is therefore not surprising that almost all of the patients treated with viral agents developed high titers of neutralizing antibodies following the first administration of the virus. Preclinical studies suggest that the presence of such antibodies might reduce the efficacy of adenoviral treatments (112). At this point, this has not been clearly demonstrated clinically. In contrast, following intra-arterial infusion of ONYX-015 into the hepatic artery or intravenous administration, the virus was cleared rapidly from the bloodstream. Pharmacokinetic analysis of the presence of viral DNA in plasma revealed a biphasic course of plasmatic levels with $t_{1/2\alpha} = 14$ minutes and $t_{1/2\beta} = 113$ minutes (74). No differences in the pharmacokinetic parameters between the first and third treatment cycles (after increases in anti-adenovirus antibody levels, see below) were observed. Nevertheless, novel concepts have been developed to address this potential problem. For example, clinical studies are being considered to test the utility of rituximab, a recombinant anti-B-cell antibody that prevents antibody induction by depleting B-cells (113), for preventing the anti-adenoviral immune response. Another strategy involves patient “desensitization” by administering first low doses of the vector followed by an intra-individual dose escalation, as it has been successfully demonstrated in clinical trials of reovirus.

Improving Tumor Killing by Improving Tumor Cell Access

Viral vectors and oncolytic agents have been engineered to improve their ability to infect cancer cells, either to increase selectivity or to increase potency. Adenoviruses have been the focus of many of these efforts. This is because it is believed that the utility of adenovirus vectors is limited due to the low expressing of CAR, the high-affinity receptor that is necessary for efficient attachment to the cell membrane (114). This is of particular concern in many advanced cancers, in which CAR levels are often low relative to normal cells or well-differentiated cancers (115,116). Many attempts have been made to address this problem (Figure 56-2). Wickham et al. (117) modified the C-terminus of the adenoviral fiber protein by the addition of an RGD-containing peptide or the addition of seven lysine residues. Dmitriev et al. (118,119) have also shown that the incorporation of an RGD-containing peptide in the H1 loop of the fiber knob domain results in the ability of the virus to utilize an alternative receptor during the cell entry process. The modified virus was able to infect primary tumor cells and tumor cell lines more efficiently than unmodified virus (118,120). The RGD/fiber modification was subsequently introduced into the Delta-24 virus, described previously. Gu et al. (121) have successfully redirected cell binding and uptake of an adenovirus through fibroblast growth factor receptors (FGFRs), suggesting that redirecting the native tropism of adenovirus may offer therapeutic benefit.

An alternative strategy to improve access to tumor cells for adenoviruses is to pharmacologically restore receptor expression. CAR is a cell-cell adhesion molecule that is regulated in its expression through the Raf-MEK-ERK and TGF- β pathways (122,123). Treatment of tumor cells with inhibitors of these pathways can increase virus entry into and killing of tumor cells (Figure 56-2). Similarly, treatment of tumor cells with low CAR expression with histone-deacetylase inhibitors increases CAR levels, infectability, and antitumor effects of therapeutic adenoviruses in vitro and in vivo (124,125). It is noteworthy, however, that alterations in tissue architecture, for example the partial loss of tight cell–cell adhesions, might render tumor cells more susceptible to virus infection (126).

Conclusion and Future Prospects

The first goal of this field was to invent and test new agents that kill cancer cells selectively, based on the genetic lesions that cause this disease. This goal has been achieved using a rich variety of viruses, vectors, genes, and approaches. Some of these approaches have been translated into clinical research projects, but none has yet been proven to result in increased survival or meaningful patient benefit. For the most part, high doses of therapeutic agents have been well tolerated, but at these high doses, clinical activity has generally been lacking. Higher doses that might prove beneficial are expected to be toxic. We may therefore conclude that although we are able to kill cancer cells with very high selectivity in the laboratory, we are unable to achieve a satisfactory therapeutic index in vivo. This lack of compelling clinical activity, together with the potential risk and cost of manufacturing these agents, has

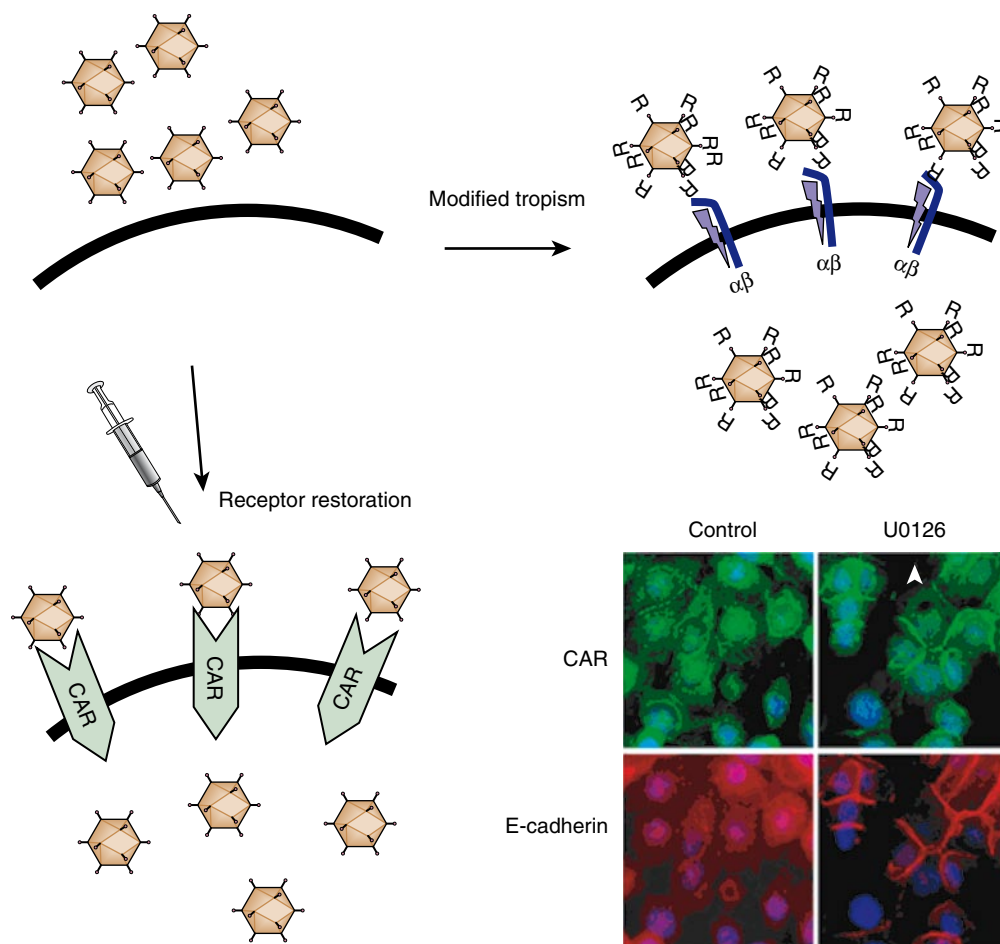


FIGURE 56-2 Approaches to increasing adenovirus infectivity. The primary receptor for adenovirus, CAR, is often down-regulated in cancer cells, resulting in poor infectivity. This can be reversed using genetically modified viruses with altered tropism (for example, an RGD motif to increased binding to surface integrins, as in Delta-24 RGD) or by pharmacologic intervention. In this example, cancer cells are treated with a MEK inhibitor, U0126, which results in redistribution of CAR (and E-cadherin) to the cell surface, and increased infectivity. (From Anders M, Christian C, McMahon M, et al. Inhibition of the Raf/MEK/ERK pathway up-regulates expression of the coxsackievirus and adenovirus receptor in cancer cells. *Cancer Res* 2003;63(9):2088–2095, with permission.)

kept interest in this field in the pharmaceutical industry at a low level. As a result, significant investments have not been made in developing methods for scaling up and manufacturing or in clinical development in general.

The major issue that is responsible for the lack of therapeutic efficacy *in vivo* appears to be poor access to tumor cells. When viruses are injected directly into tumor cells, they appear to penetrate tumors inefficiently. Viruses that replicate in tumor cells do not spread effectively either. Systemic treatment results in clearance by the liver and other tissues, so that low doses reach

their target. In addition, inflammatory effects of viral particles can become limiting or life threatening. Finally, repeated doses of typical viral vectors leads to an immune response that diminishes biological potency.

All of these issues represent serious challenges. However, it seems likely that the same creative efforts that enabled invention of tumor-selective agents in the first phase of this field's development could be harnessed to address each of these challenges. Some solutions have already been discussed and are under evaluation.

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RNA as a Therapeutic Molecule

Cancer represents the most complex genetic disease. Despite of tens of years of investigation and the use of a huge amount of resources, the mortality by cancer did not decrease significantly worldwide, particularly in developed countries (1). Many explanations were offered, but the basic point is that we do not yet deeply understand the mechanisms by which tumorigenesis occurs. Therefore, there are major reasons for developing new therapeutic modalities to cure cancer. Recently a new layer of genetic complexity was discovered in malignant cells by the addition of the noncoding RNAs (ncRNAs; RNAs that do not codify for proteins) on the list of cancer genes, and exploiting this research could provide a great treatment opportunity. Globally, the death rates from all cancers combined have decreased only slightly in the last 12 years (by 16.3% in men and by 8.5% in women), largely because of the advances in early detection of breast cancer and of the reduction in tobacco use over the past four decades, while the therapeutic advances were less significant. For example, the oligoantisense strategy was considered for many years as an optimal alternative for developing drugs to inhibit the proteins overexpressed in cancer cells (for a review, see [2]). This is the case of the BCL2 antisense oligonucleotide (ASO; 3,4), but until now no other ASO agents proved consistent and reproducible benefic effects in clinical trials. Six publication in the last 4 years reported clinical studies regarding trials in phases 2 (to determine whether a new treatment work) or 3 (to study whether a new treatment is better than standard treatment) performed on 828 patients by using Aprinocarsen (Affinitak, LY 900003, ISIS 3512; Isis Pharmaceuticals, Carlsbad, CA; 5–10). This is a 20-mer oligonucleotide acting as an ASO that binds to the 3' untranslated region (3'UTR) of the human messenger RNA for protein kinase C α . It acts by forming an mRNA-ASO duplex through Watson-Crick binding, and it leads to RNase-H-mediated cleavage of the PKC α mRNA. In all trials, except one, no significant effects were identified in patients with advanced non-small cell lung cancer, metastatic colorectal cancer, or relapsed low-grade non-Hodgkin lymphoma.

Cancer as a Genetic Disease of Protein-Coding Genes and Noncoding RNAs

Why the need for RNAs as therapeutic molecules? One of the most unexpected and fascinating discoveries in the last few years

in molecular oncology is that in a specific tumor, abnormalities in both protein-coding genes (PCGs) and ncRNAs can be identified, and the interplay between them are causally involved in the initiation and progression of human cancers (11–13). The “classic” molecular oncology dogma was that cancer is a genetic disease involving tumor-suppressor and oncogenic proteins. Recent findings that small non-coding RNAs named microRNAs (miRNAs) are involved in the pathogenesis of most cancers reveal a new layer of complexity in the molecular architecture of human cancers (Figure 57-1).

MicroRNAs represent a new class of small ncRNAs able to regulate gene expression (14,15). MicroRNAs are distinct from, but related to small-interfering RNAs (siRNAs), which have been identified in a variety of organisms (for reviews see [15,16]). These small 19- to 24-nucleotide (nt) RNAs are transcribed as long primary transcripts of several kilobases (kb) in length, named pri-miRNA. Pri-miRNAs are processed in the nucleus into precursor hairpin RNAs (70–100 nt in length) named pre-miRNA by the double-stranded RNA-specific ribonuclease Drosha (17). The hairpin RNAs are transported to the cytoplasm, via an exportin-5-dependent mechanism, where they are digested by a second double-stranded specific ribonuclease named Dicer. In animals, single-stranded miRNAs bind specific messenger RNA (mRNA) through a low complementary binding site located in the target mRNA, mainly at their 3' UTR (14). By a mechanism that is not fully characterized, the bound mRNA remains untranslated, resulting in reduced levels of the corresponding protein and/or the bound mRNA can be degraded, causing a decrease of the transcript and consequently, of the corresponding protein. The role of miRNAs was proven to be important in essential biologic processes for the eukaryotic cell such as pancreatic cell insulin secretion (miR-375), adipocyte development (miR-375), cell proliferation control (miR-125b and let-7), brain patterning (miR-430), hematopoietic B lymphocytes lineage fate (miR-181), or B lymphocytes survival (miR-15a and miR-16-1).

MicroRNAs were found to be involved in the pathophysiology of all types of analyzed human cancers (18). Among the new paradigms of molecular oncology are the following:

1. Several genome-wide profiling techniques (for review see [13,19,20]), such as oligonucleotide miRNA microarray bead-based flow cytometric technique, quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR) for precursor and active

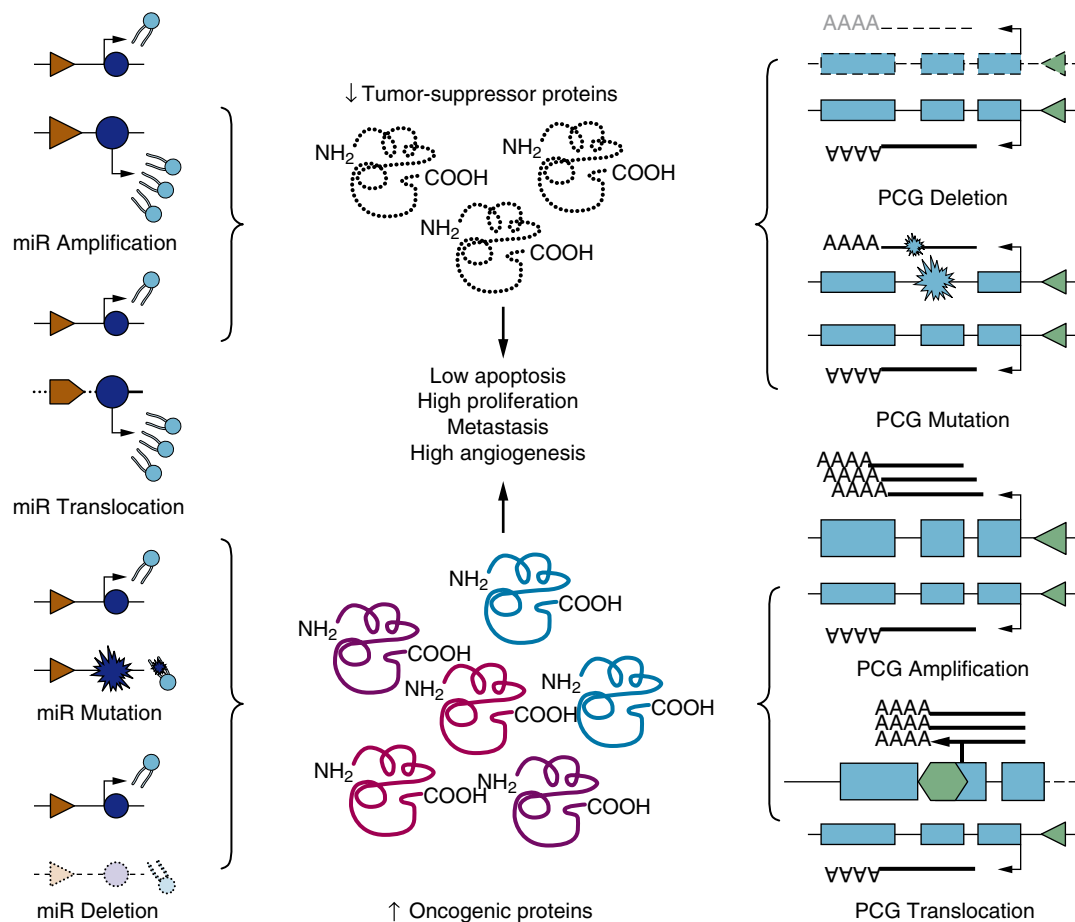


FIGURE 57-1 A CANCER MODEL INVOLVING PROTEIN CODING GENES AND NONCODING RNAs ACTING AS ONCOGENES AND TUMOR SUPPRESSORS. The main mechanisms common for microRNAs (miRNAs) and cancer-specific protein-coding genes (PCGs) misfunction in cancer cells are represented by chromosomal translocation/rearrangement, genomic amplification, biallelic mutations, deletion/promoter methylation plus mutation and biallelic deletions/promoter methylations or combination. The promoter regions are presented as triangles and the structural genes as circles (miRNAs) or rectangles (PCGs).

miRNA or the miRAGE (serial analyses of gene expression for miRNAs), were performed on various cancer histotypes, including chronic lymphocytic leukemia (CLL), breast cancer, glioblastoma, thyroid papillary carcinoma, hepatocellular carcinoma, lung cancer, colon cancer, and endocrine and exocrine pancreatic tumors. From these studies it has become clear that in cancer cells the main alteration of the micro-RNoma (defined as the full complement of microRNAs present in a genome) is represented by aberrant gene expression, consisting of abnormal levels of expression for mature and/or precursor miRNA sequences compared with the corresponding normal tissues.

2. Germ-line and somatic mutations in miRNAs (21) or polymorphisms in the messenger protein coding RNAs targeted by miRNAs (22) may also contribute to cancer predisposition, initiation, and progression. If occurring in somatic cells, miRNA alterations could initiate or contribute to tumorigenesis, although if present in the germ-line could represent cancer predisposing events.
3. MicroRNAs profiling achieved by various methods has allowed the identification of signatures associated with diagnosis, staging, progression, prognosis, and response to treatment of human

tumors (for a review see [13]). Therefore, miRNA “fingerprinting” represents a new addition to the diagnostic and prognostic tools to be used in medical oncology.

Other types of ncRNAs (such as ultraconserved genes, UCGs) were recently proved to be linked to human cancers. One of the most intriguing characteristics of miRNAs is the near-complete conservation of orthologous genes. For example, the active molecules of *miR-16-1/miR-15a* cluster, shown to be an essential player in the initiation of CLL (21), are completely conserved in humans, mice, and rats and highly conserved in nine of ten primate species sequenced (23). Comparative sequence analysis represents an essential tool in the identification of genomic DNA regions with important biologic functions. Several of these highly conserved genomic sequences were considered not genic (not producing a transcript) and were called “conserved nongenic sequences” (24). A special subset of conserved sequences named “ultraconserved regions” (UCRs) include, by definition, intra- and intergenic sections of the of human genome that are absolutely conserved (100% identity with no insertions or deletions) between orthologous regions of the human, rat, and mouse genomes (25). Because of the high degree of conservation, the UCRs have been demonstrated to have fundamental functional importance for

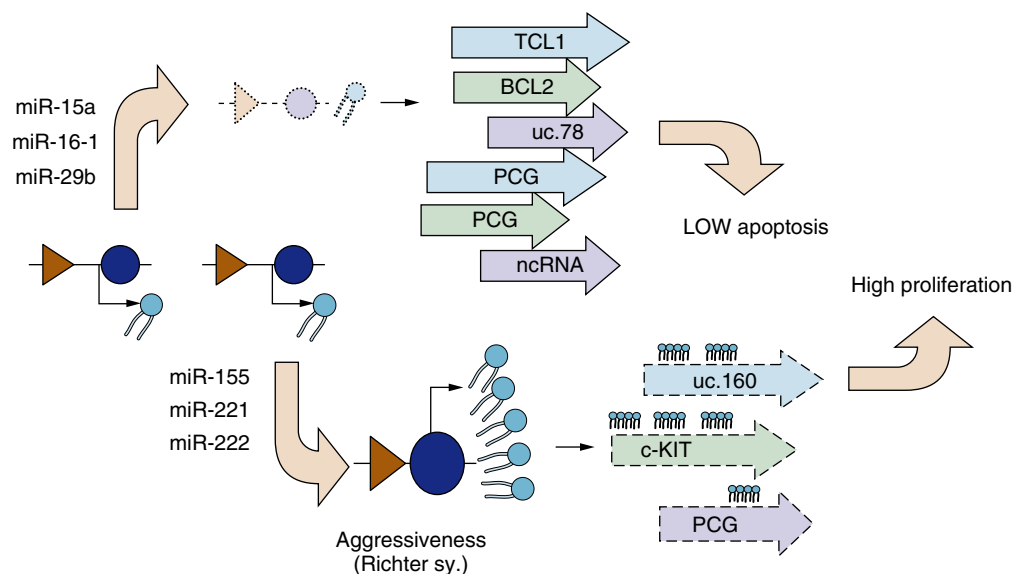


FIGURE 57-2 CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) AS THE BEST KNOWN EXAMPLE OF INTERPLAY BETWEEN NONCODING RNAs (ncRNAs) AND PROTEIN CODING GENES (PCGs) AND THE THERAPEUTIC IMPLICATIONS. During the initiation and progression of B-cell CLL alteration in at least three different types of genes, PCGs, microRNAs (miRNAs), and ultraconserved genes (UCGs) were identified. These are not independent, as complex regulatory interactions between miRNAs and PCGs and between miRNAs and UCGs occur; furthermore, we postulate the existence of interactions between UCGs and PCGs, although these were not yet demonstrated.

the ontogeny and phylogeny of mammals and other vertebrates. Recently, it was proved that most UCRs are transcribed and that hundreds of ultraconserved genes (UCGs) are consistently altered in a significant percentage of analyzed leukemias and carcinomas. UCGs are frequently located at fragile sites and genomic regions involved in cancers. It has also been proven that the inhibition of an overexpressed UCG induces apoptosis in colon cancer cells, and that the expression of some UCGs may be regulated by miRNAs abnormally expressed in CLL (26). These new regulatory mechanism support for a model in which various types of noncoding genes are involved and cooperate with protein-coding genes in human tumorigenesis (Figure 57-2). By gathering all these notions together, it is clear that noncoding RNA genes, once seen as second-level genomic elements, are now at the center of attention in cancer research. Therefore, it is reasonable to attempt to expand the anti-cancer ammunition with RNA molecules capable of attenuating or completely abolishing the function of overexpressed ncRNAs or, alternatively, to re-express at physiologic levels the deleted or down-regulated ncRNAs.

Main Types of Therapeutic RNA Molecules

Two different types of RNA molecules, the ribozymes and the small-interfering RNAs (siRNAs), have passed the preclinical testing for efficiency in down-regulating a target and now are going into the clinical trial age. Two more types of RNA molecules, the anti-miRNA agents and the miRNA-mimics agents, recently added to the expanding list of anticancer ammunition, are going through preclinical tests. The antisense oligonucleotides strategy (ASO) was primarily developed using DNA molecules (for detailed discussion, see [2,27]). Although the history of RNAs as therapeutic molecules is two decades long, few clinical trials have been conducted yet on large numbers of cancer patients to be able to draw any convincing conclusion. The first hints are encouraging and support the development of new and larger clinical trials. Most

of the clinical and preclinical data were gathered from patients with viral infections, such as human immunodeficiency virus type-1 or chronic hepatitis C virus (HCV). For a glossary of terms used in RNA inhibition strategies see Table 57-1.

Ribozymes

In 1982, the first two ribozymes, the self-splicing intron of the tetrahymena pre-rRNA and the RNaseP were discovered by Cech's group (28) and Altman's group (29), respectively. Both shared the Nobel Prize in Medicine in 1989 for this discovery. A naturally occurring or a laboratory-prepared RNA enzyme, ribozyme, is an RNA molecule that can catalyze a chemical reaction of substrate cleavage. The mechanism of action consists on three steps that are cyclically repeated: The first is represented by the Watson-Crick base pairing to a complementary target sequence, the second by the site-specific cleavage of the substrate, and the final one by the release of the cleavage products. Although there are now seven naturally occurring classes of ribozymes (30), the most commonly used class of ribozyme as therapeutic agents are the hammerhead (Hh) ribozymes. The Hh's are short-length RNAs, not more than 40 nt long and are made of two substrate-binding arms plus a conserved catalytic domain of 24 bases (31).

Angiozyme (RPI.4610; Sirna Therapeutics Inc, Boulder, CO), an angiogenesis inhibitor, is an Hh ribozyme targeting a conserved region of human vascular endothelial growth factor receptor (VEGFR-1), selectively down-regulating the VEGFR-1 by cleavage of its mRNA. It is the first synthetic ribozyme to be tested as a therapeutic agent in human cancer. Angiozyme was used in a phase 1 clinical trial as single agent in patients with biopsy-proven refractory solid tumor and showed promising results. The disease was stable in 25% of 28 eligible patients for a period of more than 6 months, with the longest treatment duration of more than 16 months and showed no significant adverse reactions (32). In another study, the same drug was combined with carboplatin and paclitaxel indicating that this multidrug regimen can be administrated in patients with advanced solid tumors with no

Table 57-1 Glossary of Terms in RNA-Inhibition Strategies

ASO: An antisense oligonucleotide is a single-stranded, chemically modified DNA-like molecule that is 17 to 22 nt in length and designed to be complementary to a selected messenger RNA and thereby specifically inhibit expression of that gene.
Messenger RNA (mRNA): The form of RNA that mediates the transfer of genetic information from the cell nucleus to ribosomes in the cytoplasm, where it serves as a template for protein synthesis. It is synthesized from a DNA template during the process of transcription.
Noncoding RNAs (ncRNAs): Any RNA molecule that is not translated into a protein.
Open Reading Frame (ORF): A section of a sequenced piece of DNA that begins with an initiation (methionine ATG) codon and ends with a nonsense codon. ORFs all have the potential to encode a protein or polypeptide, however many may not actually do so.
Phase 3 Clinical Trials: Designed to study whether a new treatment is better than standard treatment by including hundreds of patients in the study or control groups each.
Phases 2 Clinical Trials: Designed to determine whether a new treatment work by including tens of patients in the study or control groups each.
Pol II: RNA polymerase II (also called RNAP II) catalyzes the transcription of DNA to synthesize precursors of mRNA and most small nuclear RNA.
Pol III: RNA polymerase III (also called RNAP III) transcribes DNA to synthesize ribosomal 5S rRNA, tRNA and other small RNAs. The genes transcribed by RNA Pol III fall in the category of “housekeeping” genes whose expression is required in all cell types and most environmental conditions.
Sense/Antisense: Referring to the strand of a nucleic acid that directly specifies the product or referring to the strand of a double-stranded molecule that does not directly encode the product but is complementary to it.
Transcription: The process whereby RNA is synthesized from a DNA template.
Translation: The process of protein synthesis whereby the primary structure of the protein is determined by the nucleotide sequence in mRNA. The ribosome-mediated production of a polypeptide whose amino acid sequence is derived from the codon sequence of an mRNA molecule.
Untranslated Region (UTR): 5′ UTR is the portion of an mRNA from the 5′ end to the position of the first codon used in translation. The 3′ UTR is the portion of an mRNA from the 3′ end of the mRNA to the position of the last codon used in translation.
Watson-Crick Pairing: The A-T and G-C pairing between the four types of DNA nucleotides.

substantial pharmacokinetic interactions (33). The most common adverse effects were neutropenia, thrombocytopenia, pain, anemia, and fatigue. Angyozyme was well tolerated after intravenous (IV) infusion or a single subcutaneous (s.c.) bolus in healthy volunteers (34). Combined with the data from the phase 1 clinical trial, new studies will be designed and conducted to assess the efficacy in various human cancers. Furthermore, as inhibition of either VEGF-R1 or VEGF-R2 signaling can only partially block tumor angiogenesis and growth, simultaneous inhibition of VEGF-R1 and VEGF-R2 signaling could be highly effective in retarding the growth of some tumors (35).

One obvious question is why, in spite of the years-long effort to decipher the structure and function of ribozymes, are data accumulated in cancer patients so scarce? A reason is the sequence specificity requirements of targeted RNAs that limit the amount of putative targets: for example, the Hh ribozymes work at their best on GUC and AUG triplets. Another reason is the limited accessibility of the drug to the messenger RNA complementary sequence due to the internal base pairing, producing secondary structures, and to the proteins that physically associate with the RNA. Furthermore, differences in targeted messenger RNA half-lives could affect the efficacy of the silencing, being easier to eliminate a target with a short half-life than targets with much longer half-life.

To improve the efficiency of ribozymes in cancer cells, a hybrid construct was produced and referred to as maxizyme (36). This is a dimer of minimized ribozymes (minizymes) that can cleave two target sites located in two different messenger RNAs. The maxizyme also can allosterically cleave the target RNA only

when it recognizes two target sites. For example, two distinct oncogenes, cyclinD1 (*CCND1*) and fibroblast growth factor 4 (*FGF4*, named also *HST-1*), which are overexpressed in breast cancer cells, were used as targets of a maxizyme. *CCND1* activity is required for cell cycle G₁/S transition, whereas *FGF4* is involved in tumor growth and invasion, and therefore blocking these genes could target a wide spectrum of pathways in malignant cells. When conventional ribozymes were used for suppression of expression of those genes, mRNAs in cancer cells and in normal cells were affected, whereas the *trans*-maxizyme cleaved these mRNAs only in the breast cancer cells. If such a drug has similar results in clinical trials in patients with breast cancer is still an open question.

siRNAs and shRNAs

In 1998 Mello and Fire discovered RNA interference (RNAi) in vertebrates (37), for which they received the Nobel Prize in Medicine in 2006. RNAi is a form of posttranscriptional gene silencing (PTG) in which double-stranded RNA (dsRNA), named siRNA, catalyzes the degradation of complementary mRNA targets. A siRNA is a double-strand (ds) RNA homologous to an mRNA of a target gene. In cytoplasm, the dsRNAs are processed by a complex consisting of Dicer and several other proteins into siRNAs, which are loaded into Argonaute 2 and RNA-induced silencing complex (RISC). The siRNA guide strand recognizes target sites to direct mRNA cleavage, which is carried out by the catalytic domain of AGO2. The processing of the siRNAs is similar of that of miRNAs which is viewed as the “endogenous” process of RNA duplexes maturation (38,39).

A small-hairpin RNA (shRNA) represents an siRNA-like molecule expressed from a vector (40). DNA cassettes encoding RNA polymerase III promoter-driven siRNA-like shRNAs allow long-term expression of therapeutic RNAs in targeted cells. One advantage over the ribozyme technology is the higher efficiency in targeting specific messengers. For example, when the efficiency of the shRNAs was compared with adenoviral delivery of an anti-MDR1 (multidrug resistance) ribozyme construct, the shRNAs' down-regulation of mRNA and protein expression were accompanied by a complete inhibition of the pump activity of MDR1 and a reversal of the multidrug-resistant phenotype. The ribozyme construct weakly affects gene expression, confirming that adenoviral delivery of shRNAs is much more effective than adenoviral delivery of ribozymes and that adenovirus-based vectors can be very powerful agents for efficient delivery of therapeutic RNA molecules (41).

Oncogenes expressed at abnormally high levels represent the main targets of siRNA-directed therapy. The results from preclinical studies are promising and show clear efficacy. For instance, in a mouse model of ovarian cancers overexpressing the tyrosine kinase receptor *Eph2* gene, the administration of liposomal-delivered siRNA targeting *Eph2* combined with paclitaxel determined a reduction of the tumor size greater than 50% with intravenous or intraperitoneal routes of delivery. These data support the idea that chemotherapy and siRNA together can provide a powerful anticancer combination (42). In another example, adenovirus-mediated siRNA against a *K-ras* mutated messenger (*K-ras* codon 12 GGT to GTT) markedly decreased *K-ras* gene expression and inhibited cellular proliferation of lung cancer cells that express the relevant mutation, but produced only minimal growth inhibition on cells that lack the specific abnormality (43).

Although there are promising preclinical data, siRNA cancer therapy is overshadowed by few, but significant, concerning issues. The first involves the low bioavailability: in aqueous solution siRNAs are extremely hydrophilic and heavily hydrated because of the sugar-phosphate backbone exposed to the water, therefore reducing their diffusion to the target tissue. Furthermore, because of the degradation by serum nucleases, the *in vivo* half-lives for siRNAs are short. Chemical modifications of siRNAs to overcome these deficiencies include the conformationally "locked" nucleotide analog (LNA), which substantially increases the serum stability, without adversely affecting interactions with cellular silencing machinery. Introduction of a 2'-O-methyl group (2'-O-Me) and 2'-fluoro nucleotides enhances plasma stability and increases potency hundred of times *in vivo* over the unmodified, "naked" siRNAs (44). Another unsolved problem is represented by "off-target-effects" (OTEs), meaning that, in addition to the complementary target, a specific siRNA can induce the silencing of several other imperfect complementary messenger RNAs that can be important for cell homeostasis. OTEs have been demonstrated by transcriptional profiling studies, and the nonspecific genes differentially expressed between treated and nontreated cells contain complementary regions to one of the two strands in the siRNA duplex. Chemical modification of nucleotides within

this region of homology, by the introduction of a 2'-O-Me group, reduces the OTE without decreasing the silencing activity on messenger RNAs. Finally, a major concern is the stimulation by siRNA duplexes of the innate immune system and the production of high amounts of interferon. Sugar modifications (such as 2'-O-Me) and locked nucleic acids (LNAs) seem to reduce the immunostimulatory effects of siRNAs (for a comprehensive review on this topic see [44]).

ASOs/AMOs Anti-miRNAs, LNAs Anti-miRNAs, and Antagomirs

The rationale for considering miRNAs as potential therapeutic targets is offered by the fact that miRNA overexpression in cancer cells has a pathogenic effect (see preceding sections). Therefore, several types of agents targeting miRNAs are now under development and ready to be tested for their *in vivo* effects and in clinical trials (45,46).

Anti-miRNA oligonucleotides (AMOs) represent antisense oligonucleotides (ASOs), single-stranded, chemically modified DNA-like molecules that are 17 to 22 nt in length and designed to be complementary to a selected miRNAs. Thus they are able to specifically inhibit expression of that gene. Mechanistically, the AMOs can be described as ASOs against miRNAs, and therefore produce ASO-miRNA duplex through Watson-Crick binding, leading to RNase-H-mediated cleavage of the target miRNA gene. Important for the potential clinical use, ASOs harboring a complete 2'-O-methoxyethyl and phosphorothioate modification have been demonstrated to silence *in vivo* *miR-122* in mouse liver (47). The LNAs anti-miRNAs represent modified antisense single-stranded oligonucleotides 17 to 22 nt in length, with a methylene bridge connecting the 2' and the 4' carbones. *miR-21*, shown to be strongly overexpressed in glioblastomas, was silenced *in vitro* by using LNA-modified antisense oligonucleotides leading to a significant reduction in cell viability and elevated intracellular levels of caspases (48).

The antagomir represents RNA therapeutic molecules originally designed to inhibit the miRNAs (49,50). These are chemically modified and cholesterol-conjugated single-stranded 23-nt RNA molecules complementary to the targeted miRNAs. The modifications were introduced to increase the stability of the RNA and protect it from degradation. When intravenously administered to mice, antagomirs against *miR-122* (antagomir-122), a miRNA highly expressed in liver induced a marked, specific, and persistent (up to 23 days) reduction of endogenous miRNA gene expression. The same was true for antagomir-16, targeting the ubiquitously expressed *miR-16*. Silencing of miRNAs by these new agents was followed by physiologic effects, for example the decrease in plasma cholesterol levels after antagomir-122 administration. The only tissue where antagomirs did not act when injected systemically was the brain, probably due to the difficulty of crossing the blood-brain barrier, but they efficiently targeted miRNAs when injected locally into the mouse cortex. One clear advantage with respect to siRNA technology is that antagomirs did not induce an immune response.

miR-Mimics Agents

There are no reports in clinical practice of using the mimic miRNA agents that reproduce the effects of endogenous miRNAs and include all the chemical modifications necessary for stability and processing. However, the restoration of the expression of specific miRNAs abnormally expressed because of genomic deletions, hypermethylation, or other causes could be clinically used in the next future. For example, lung carcinomas represent the leading cause of cancer deaths in men worldwide; the identification of new therapeutic agents could represent a significant advance. The *let-7* family of miRNAs is highly down-regulated in lung cancers (51) and reduced *let-7* expression was found significantly correlated with shorter survival after potentially curative resection (52). Because *let-7* negatively regulates *RAS*, and *RAS*-p21 protein overexpression is associated with a worse survival (53), administration of *let-7* agents in patients with lung cancer might be a new avenue of treatment.

In the Search of the Right Way and the Right Type of Delivery

Two practical issues regarding the use of RNAs as therapeutic molecules should be further dissected: the selection of the most adequate formula and the most efficient way of administration (local vs. systemic): naked RNA delivery or vehicle delivered and viral vehicle versus nonviral delivery are the most important questions in the formula type debate. Initial therapeutic applications used 21-nt siRNA duplexes, with 2-nt 3' overhangs, as well as longer siRNAs of 27 mers and shRNAs (29 nt). These drugs are able to induce only transient gene silencing, since their intracellular concentrations diminish with every cell division (38). However, despite improvements in siRNA stability, naked siRNAs are of

little therapeutic use and are likely to require some form of targeted delivery vehicle for sufficient tissue specificity and cellular uptake. Long-lasting and stable knockdown of target transcription is achieved by expression of shRNAs from various types of vectors, including viral vectors, with Pol II or Pol III promoters. Although therapeutically useful, the continuous expression can cause harmful side effects due to the saturation of the endogenous silencing pathway; thus abundant shRNA expression could be toxic. For example, the use of an adeno-associated virus (AAV) vector with a U6 promoter (Pol III type) induced high expression levels of shRNAs and caused death in about half of the treated mice (54). Different lentiviral vectors (LVs), adenoviral vectors (AVs) and AAVs are being tested. LVs efficiently integrate into the genome of nondividing cells, such as pancreatic islets or terminally differentiated cells. In contrast to LVs, AVs do not integrate into the host genome they do however efficiently transduce dividing and nondividing cells. Different AAV serotypes can be potentially used for organ-directed miRNA expression. Another option is to use nonviral delivery vehicles for shRNAs. Liposomes, lipid complexes with small molecules, polymers, proteins, and antibodies were tested as potential delivery options and all have certain advantages (but also clear disadvantages; 55). With these delivery partners the efficacy of shRNA is increased and doses reduced, but the cytotoxic effects are much higher (Table 57-2).

A second practical issue is the selection of local versus systemic administration of RNA drugs. Local administration has certain advantages, such as the need for lower doses with consequent lower adverse/toxic reactions and better bioavailability of the drug to the target tissue. Numerous publications have described the delivery of siRNA molecules directly to tumors in vivo, including direct applications of siRNA into to the peritoneal cavity, testes, or urethra (reviewed in [56]). In other instances, such as leukemias, local administration cannot be achieved and therefore systemic delivery is mandatory.

Table 57-2 Delivery Methods for RNA-Interference–Based Therapeutics

Method	Molecule Delivered	Advantage	Disadvantage
Nonviral Delivery			
Cholesterol	siRNA	Systemic delivery, stable	Nonselective delivery
SNALP	siRNA	Systemic delivery, highly stable	Nonselective delivery
Fab	siRNA	Selective delivery	Relatively complex formulation
Aptamer	siRNA	Selective delivery	Large-scale sequence screening required
Nanoparticle	siRNA	Receptor-specific, self-assembling	Sophisticated preparation required
Viral Delivery			
Lentivirus	RNA (shRNA produced)	Stable expression, transduces nondividing cells	Gene-disruption risk, localized delivery
Adenovirus	dsDNA (shRNA produced)	Episomal, no insertional mutagenesis	Immunogenic, dose-dependent hepatotoxicity, transient expression
AAV	ssDNA/dsDNA (shRNA produced)	Episomal, low genomic integration	Immunogenic, small sector capacity, transient expression

dsDNA, double-strand DNA; SNALP, stable nucleic acid-lipid particle; ssDNA, single-strand DNA. (Modified with permission after Kim and Rossi, 2007, Nature Review Genetics.)

A Strategy for Using RNA as Therapeutic Molecules

RNA molecules are now at the center of molecular oncology, with applications for diagnosis and therapy starting to be proposed. Although exciting and full of promises, the field of RNA as a therapeutic molecule is not free of obstacles. Therefore, a good practical option is to attempt to improve the strategies to optimize the efficiency of this new anticancer tool in the light of the non-fulfilled promises of other types of new therapies. For example, to highlight, let us use the example of B-cell chronic lymphocytic leukemia (B-CLL), the most frequent adult leukemia in the Western world (57) and one of the best studied models of interplay between coding and ncRNAs in human cancers (21). B-CLL is characterized by predominantly nondividing malignant B cells

overexpressing the anti-apoptotic Bcl2 protein. Deletion or down-regulation of *miR-15a/miR-16-1* cluster located at chromosome 13q14.3 represents an early event directly involved in the regulation of BCL2 expression (58). During the evolution of malignant clones, other miRNAs are deleted (such as *miR-29*) or overexpressed (such as *miR-155* or the cluster *miR-221/miR-222*) contributing to the aggressiveness of B-CLL (Richter syndrome). Such abnormalities influence the expression of other protein-coding genes: reportedly *miR-221* and *miR-222* directly down-modulate *c-KIT* receptor and *miR-29* regulates the levels of TCL1 oncogene (59) or targets other ncRNAs such as UCGs *uc.160* and *uc.78* (Figure 57-2; 26). The consequences of this steady accumulation of abnormalities are represented initially by low apoptosis and high survival of malignant B cells and later by the evolution of more aggressive clones with a higher proliferative capacity and survival by the overexpression of BCL2 and TCL1 oncogenes. Therefore, a

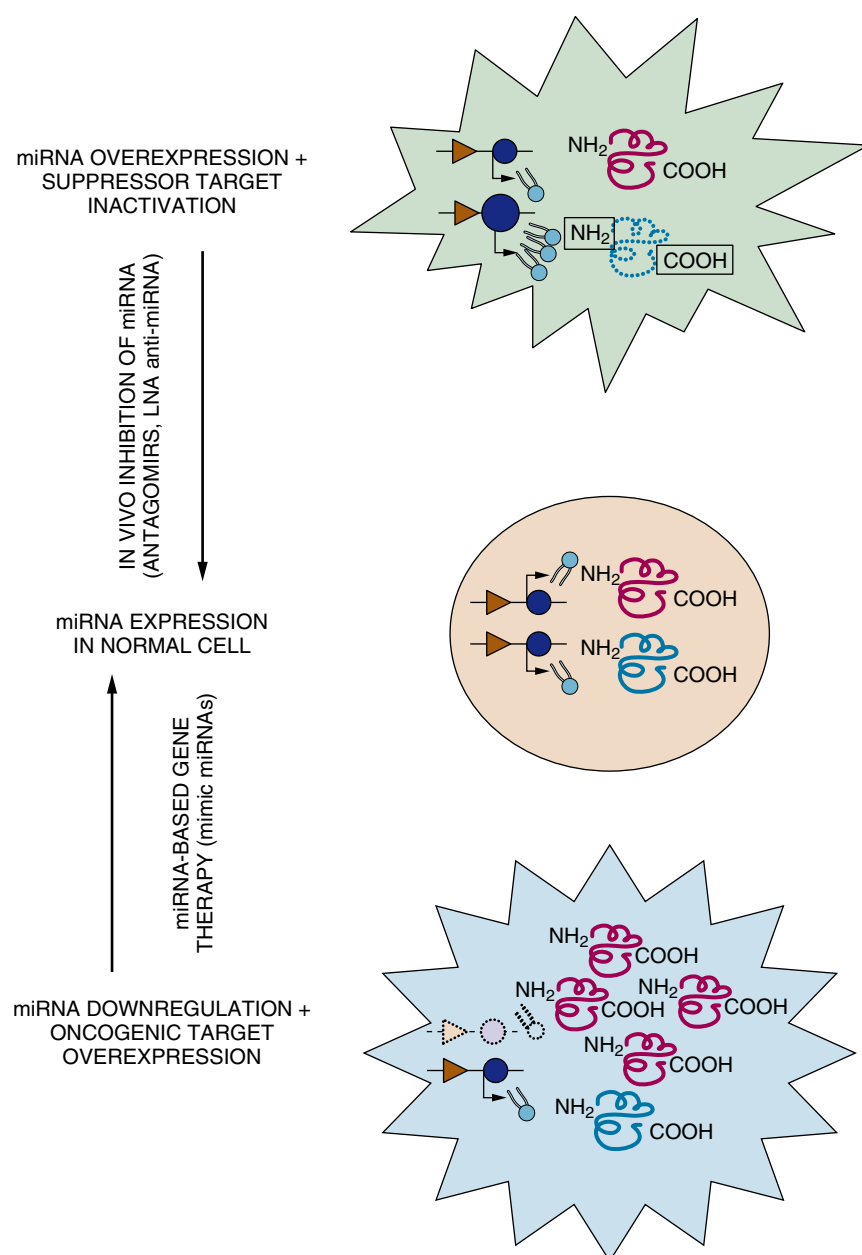


FIGURE 57-3 MICRORNAs (miRNAs) AND ANTI-miRNAs AS NEW THERAPEUTIC AGENTS. The type of therapeutic intervention is different in respect with the type of genetic alteration. If overexpressed, agents that specifically reduce to normal levels the miRNA expression should be used, while if down-regulated, agents that restore the miRNA expression have to be used. (Modified from *Pharmacogenomics* 2007;3(5): 521–537.)

mixed strategy of targeting both these oncogenes at the same time and using combined RNA drugs could be proposed. For example, designing a maxyzime anti-BCL2 and anti-TCL1 could be an option. Combining the systemic delivery of *miR-15* and *miR-16* family members targeting BCL2, together with *miR-29* and *miR-181* family members against TCL1, could increase the efficacy of therapeutic oncogenic down-regulation. Furthermore, taking advantage of the genome-wide profiling technologies, these therapies could be provided to only the subset of CLL patients having both types of lesions, specifically offered only to the patients with BCL2 overexpression and *miR-15* and *miR-16* down-regulations (the indolent form of CLL), or only to the patients with TCL1 overexpression and *miR-29* and *miR-181* down-regulation (the aggressive form of CLL). Furthermore, in the light of the newly discovered interactions between various categories of ncRNAs,

targeting not miRNAs but ultraconserved genes regulated by miRNAs starts to be an alternative option. Certainly, this is an exciting time for the RNA therapeutics and we are looking forward to the results of clinical studies investigating such strategies.

Acknowledgments

Dr. Croce is supported by Program Project Grants from the National Cancer Institute and Dr. Calin by the CLL Global Research Foundation and by an M.D. Anderson Trust grant. We thank to Drs. Milena Nicoloso and Riccardo Spizzo for the critical reading of the manuscript. We apologize to the many colleagues whose work was not cited; due to space limitations we cited mainly the significant reviews and recent clinical studies.

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Heat Shock Protein 90 and the Proteasome

Housekeeping Proteins Become Novel Molecular Targets for Cancer Therapy

Heat shock protein 90 (Hsp90) is a molecular chaperone required for the stability and function of a number of conditionally activated and/or expressed signaling proteins, as well as multiple mutated, chimeric, and/or overexpressed proteins. Regulated protein degradation, mediated by molecular chaperones, polyubiquitination, and the proteasome, is an essential mechanism for rapidly modifying cell signaling in response to environmental factors, as well as for ridding the cell of terminally misfolded proteins. Together, Hsp90 inhibitors and proteasome inhibitors are unique in that, although they are directed toward specific molecular targets (Hsp90 and the proteasome), they interfere with these basic processes and with numerous signaling networks and cell machinery upon which cancer cells preferentially depend for survival. Both Hsp90 inhibitors and proteasome inhibitors are used in the clinical setting. The efficacy of these agents may lie in their ability to circumvent, and perhaps even to take advantage of, the characteristic genetic plasticity that has allowed cancer cells to eventually evade the toxic effects of most molecularly targeted agents.

Introduction

Cancer is a disease of genetic instability. Although only a few specific alterations seem to be required for generation of the malignant phenotype, at least in colon carcinoma there are approximately 10,000 estimated mutations at time of diagnosis (1,2). This genetic plasticity of cancer cells allows them to frequently escape the precise molecular targeting of a single signaling node or pathway, making them ultimately nonresponsive to molecularly targeted therapeutics. Even Gleevec (Novartis Pharmaceuticals Corp, Basel, Switzerland), a well-recognized clinically active Bcr-Abl tyrosine kinase inhibitor, can eventually lose its effectiveness under intense, drug-dependent selective pressure due to either mutation of the drug interaction site or expansion of a previously existing resistant clone (3). Most solid tumors at the time of detection are already sufficiently genetically diverse to resist single-agent molecularly targeted therapy (4). However, such an enhanced rate of mutation suggests that cancer cells should be highly dependent on efficient chaperoning and degradation of terminally misfolded

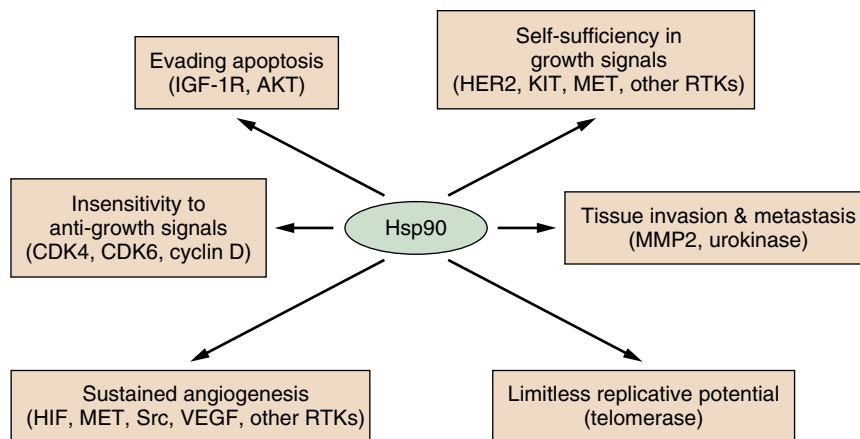
proteins, since unchecked accumulation of misfolded proteins can initiate apoptosis (5). Further, a multipronged attack on tumor cell signaling is likely to prove more efficacious than targeting individual molecular pathways. Thus, pharmacologic agents that inhibit a cancer cell's ability to dispose of misfolded protein, provide a simultaneous attack on multiple signaling nodes, or possess both properties simultaneously should be of benefit. Hsp90 inhibitors and proteasome inhibitors display these characteristics and are actively being developed as novel anticancer agents.

HSP90: A Chaperone of Oncogenes

Given the number of key nodal signaling proteins that are Hsp90 clients (see the Website maintained by D. Picard, <http://www.picard.ch/DP/downloads/Hsp90interactors.pdf>, as well as several excellent reviews [6–8]), inhibition of Hsp90 may serve the purpose of collapsing, or significantly weakening, a cancer cell's safety net. Indeed, following a hypothesis first proposed by Hanahan and Weinberg (9), genetic instability allows a cell to eventually acquire six capabilities that are characteristic of most if not all cancers. These are (1) self-sufficiency in growth signaling; (2) insensitivity to antigrowth signaling; (3) ability to evade apoptosis; (4) sustained angiogenesis; (5) tissue invasion and metastasis; and (6) limitless replicative potential. As is highlighted in Figure 58-1, Hsp90 plays a pivotal role in acquisition and maintenance of each of these capabilities. Several excellent reviews provide an in depth description of the many signaling nodes regulated by Hsp90 (10–16).

Cancer cells survive in the face of frequently extreme environmental stress, such as hypoxia and acidosis, as well as in the face of the exogenously applied environmental stresses of chemotherapy or radiation. These stresses tend to generate free radicals that can cause significant physical damage to cellular proteins. Given the combined protective role of molecular chaperones toward damaged proteins and the dependence of multiple signal transduction pathways on Hsp90, it is therefore not surprising that molecular chaperones in general, and Hsp90 in particular, are highly expressed in most tumor cells. However, Hsp90 may be elevated in tumor cells and may provide a unique molecular

FIGURE 58-1 Heat shock protein 90 (Hsp90) function is required for establishment and maintenance of each of the six hallmarks of a cancer cell, as first proposed by Hanahan and Weinberg (Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70). Importantly, Hsp90 function may also allow cancer cells to survive the genetic instability on which acquisition of the six hallmarks depends.



target therein for an additional reason. Using *Drosophila* and *Arabidopsis* as model systems, Lindquist and colleagues have shown that an ancient function of Hsp90 may be to permit accumulation at the protein level of inherent genetic mutations, and thus the chaperone may play a pivotal role in the evolutionary process itself (17,18). Extrapolating this hypothesis to genetically unstable cancer cells, it is tempting to consider that Hsp90 may be critical to their ability to survive in the presence of an aberrantly high mutation rate (19).

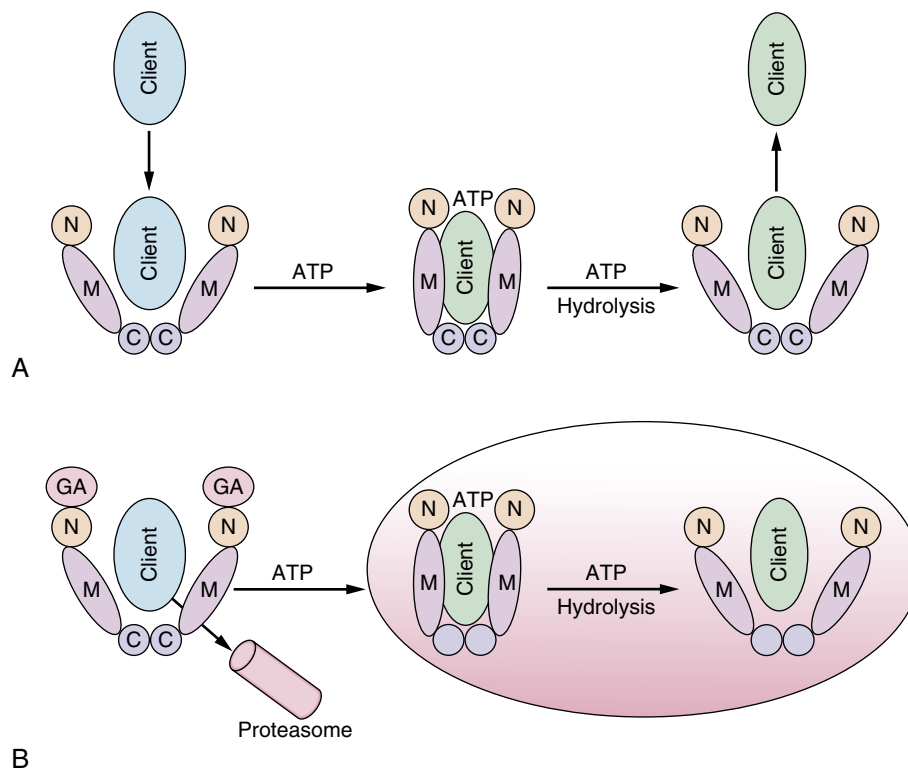
The benzoquinoid ansamycin antibiotics, first isolated from the actinomycete, *Streptomyces hygroscopicus* var. geldanus var. nova (20) include geldanamycin (GA) and its semisynthetic derivatives, 17-allylamino-17-demethoxygeldanamycin (17-AAG) and the more water-soluble 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; Figure 58-2). These small molecules inhibit the chaperone function of Hsp90 (21) and are being

evaluated in phase 1 and 2 clinical trials. The parent compound, GA, is broadly cytotoxic in the NCI-60 cell-line screen (22); its poor solubility and unacceptable liver toxicity in dogs precluded testing in humans. Because 17-AAG is less toxic than GA in rats (23) and caused growth inhibition in breast (24), melanoma (25), and ovarian mouse xenograft models, the National Cancer Institute (NCI) initiated phase 1 trials in 1999.

Several excellently detailed reviews of the mechanics of Hsp90 function are in the scientific literature (11,13,14,26–28). Hsp90 is a conformationally flexible protein that associates with a distinct set of cochaperones in dependence on nucleotide (adenosine triphosphate [ATP] or adenosine diphosphate [ADP]) occupancy of an amino-terminal binding pocket in Hsp90. Nucleotide exchange and ATP hydrolysis (by Hsp90 itself, with the assistance of cochaperones) drive the so-called Hsp90 chaperone machine to bind, chaperone, and release client proteins. Indeed, identification

FIGURE 58-2 THE HEAT SHOCK PROTEIN 90 (Hsp90)

CHAPERONE MACHINE. A: Client proteins associate weakly with an Hsp90 dimer in the absence of adenosine triphosphate (ATP). Upon ATP binding to an amino-terminal pocket in the chaperone, the N-lobes of each Hsp90 monomer transiently dimerize resulting in tight binding of the client protein to Hsp90. Upon ATP hydrolysis, stimulated by various cochaperones, dimerization of the N-lobes of Hsp90 is broken, releasing the now folded client protein. **B:** Geldanamycin (GA) and other N-terminal Hsp90 inhibitors block ATP binding to the N-lobes of Hsp90, preventing dimerization and maintaining Hsp90 in a conformation that weakly associates with client protein. In the absence of ATP binding, the client protein dissociates from Hsp90, becomes polyubiquitinated by chaperone-dependent E3 enzymes, and is ultimately degraded by the 26S proteasome.



of the GA binding site as a nucleotide pocket favoring purines led Chiosis and colleagues to design a series of highly potent purine scaffold Hsp90 inhibitors with markedly improved druglike properties (29–32). Workman and colleagues used a high-throughput screen based on inhibition of Hsp90 ATPase activity to identify 3,4-diarylpyrazoles as a novel class of Hsp90 inhibitors (33,34). By using biochemical evaluation and crystallography, these investigators found that pyrazole inhibitors of Hsp90 provide a platform for extensive derivatization and provide an attractive starting point for hit to lead exploration.

A model of Hsp90 function has emerged in which nucleotide binding to the amino terminal pocket alters Hsp90 conformation sufficiently to define distinct, nonoverlapping subsets of cochaperone proteins with which Hsp90 interacts, thus forming a “super-chaperone machine” that cycles between at least two conformations (35). An Hsp90 client protein first associates with an Hsp70/Hsp40 chaperone complex (36). This assemblage is next linked to Hsp90 via p60Hop, an Hsp90/Hsp70-interacting protein. At this point, when the client protein is being loaded on Hsp90, the chaperone is in its ADP-bound or apo conformation. Replacement of ADP by ATP in Hsp90 alters its conformation, releasing p60Hop and the Hsp70/Hsp40 complex, and recruiting another set of cochaperones, including p23 and certain immunophilins or (in the case of kinase client proteins) p50Cdc37. This association of cochaperones with Hsp90 folds and stabilizes client proteins and holds them in a metastable state that can bind ligand (e.g., steroid hormones) or respond to a stimulus (e.g., cytosolic kinases). However, this machine is neither static nor unidirectional. If a client protein fails to receive its stimulus or see its ligand while it is in the receptive conformation, the chaperone cycle will reverse. ATP hydrolysis alters Hsp90 conformation, releasing those cochaperones that associate with its ATP-bound state and recruiting those cochaperones that associate with Hsp90's ADP-bound conformation. Although the ATPase activity of Hsp90 is very weak, it is enhanced by both cochaperone association and client protein binding (37–39). Panaretou and colleagues identified a novel Hsp90 cochaperone, termed “Aha1,” that stimulates the ATPase activity of Hsp90 (40).

Thus, a highly orchestrated and tightly regulated cycle of ATP binding and hydrolysis drives the Hsp90 super-chaperone machine. When a client protein is associated with the apo- or ADP-bound Hsp90-based chaperone complex, it is not capable of responding to a stimulus or binding ligand or partner protein. The mechanism by which a client protein is targeted for degradation is not well understood, but may partly depend on the frequency with which it finds itself in the Hsp70 portion of the cycle, for it is at this point that the chaperone machine appears capable of recruiting specific ubiquitinating and proteasome-interacting proteins that serve to redirect the client protein to the proteasome, where it is degraded.

The Hsp90 inhibitors in clinical trials (17-AAG and 17-DMAG), as well as those under development, all share the property of displacing nucleotide from the amino-terminal pocket in Hsp90, and therefore short-circuiting the Hsp90 chaperone machine, much as one would stop the rotation of a bicycle wheel by inserting a stick between the spokes. Cycling of the chaperone

machine is critical to its function. The Hsp90 inhibitors, by preventing nucleotide-dependent cycling, interfere with the chaperone activity of Hsp90, and they promote targeting of client proteins to the proteasome where they are degraded (41). Even if the proteasome is inhibited, client proteins are not rescued from Hsp90 inhibition, but instead accumulate in a misfolded, inactive form in detergent-insoluble subcellular complexes (42).

Hsp90 Inhibitors Target Mutated and Chimeric Proteins Uniquely Expressed in Certain Cancers

Hsp90 characteristically chaperones a number of mutated or chimeric kinases that are key mediators of disease. Thus, anaplastic large cell lymphomas are characterized by expression of the chimeric protein NPM-ALK, which originates from a fusion of the nucleophosmin (NPM) and the membrane receptor anaplastic lymphoma kinase (ALK) genes. The chimeric kinase is constitutively active and capable of causing malignant transformation (43). Bonvini and colleagues have shown that NPM-ALK kinase is an Hsp90 client protein, and that GA and 17-AAG destabilize the kinase and promote its proteasome-mediated degradation in several anaplastic large cell lymphoma cell lines (44).

FLT3 is a receptor tyrosine kinase that regulates proliferation, differentiation, and survival of hematopoietic cells. FLT3 is frequently expressed in acute myeloid leukemia, and in 20% of patients with this cancer, the tumor cells express a FLT3 protein harboring an internal tandem duplication in the juxtamembrane domain. This mutation is correlated with leukocytosis and a poor prognosis (45). Minami and colleagues have reported that Hsp90 inhibitors cause selective apoptosis of leukemia cells expressing tandemly duplicated FLT3. Further, these investigators reported that mutated FLT3 was an Hsp90 client protein and that brief treatment with multiple Hsp90 inhibitors resulted in the rapid dissociation of Hsp90 from the kinase, accompanied by the rapid loss of kinase activity together with loss of activity of several downstream FLT3 targets including MAP kinase, Akt, and Stat5a (46). Minami et al. proposed that Hsp90 inhibitors should be considered as promising compounds for the treatment of acute myeloid leukemia characterized by tandemly duplicated FLT3 expression.

BCR-ABL (p210^{Bcr-Abl}) is an Hsp90 client protein that is also effectively inhibited by the novel tyrosine kinase inhibitor imatinib (42,47,48). While imatinib has proven very effective in initial treatment of patients with chronic myelogenous leukemia, most patients who are treated when their disease is in blast crisis stage (e.g., advanced) eventually relapse despite continued therapy (49). Relapse is correlated with loss of BCR-ABL inhibition by imatinib, due either to gene amplification or to specific point mutations in the kinase domain that preclude association of imatinib with the kinase (50). Gorre and colleagues have reported the exciting finding that BCR-ABL protein that was resistant to imatinib remained dependent on Hsp90 chaperoning activity and thus retained sensitivity to Hsp90 inhibitors, including GA and 17-AAG. Both compounds induced the degradation of “wild-type” and mutant BCR-ABL, with a trend indicating more potent activity toward mutated imatinib-resistant forms of the kinase (51). These findings were confirmed

by other investigators (52), thus providing a rationale for the use of 17-AAG in treatment of imatinib-resistant chronic myelogenous leukemia.

Mutations in the proto-oncogene *c-kit* cause constitutive kinase activity of its product, KIT protein, and are associated with human mastocytosis and gastrointestinal stromal tumors (GISTs). Although available tyrosine kinase inhibitors are effective in the treatment of GIST, there has been limited success in the treatment of mastocytosis. Treatment with 17-AAG of the mast cell line HMC-1.2, harboring the Asp816Val and Val560Gly KIT mutations and the cell line HMC-1.1, harboring a single Val560Gly mutation, causes both the level and activity of KIT and downstream signaling molecules AKT and STAT3 to be down-regulated following drug exposure (53). These data were validated using Cos-7 cells transfected with wild-type and mutated KIT. The 17-AAG promotes cell death of both HMC mast cell lines. In addition, neoplastic mast cells isolated from patients with mastocytosis and incubated with 17-AAG *ex vivo* are selectively sensitive to Hsp90 inhibition as compared with the mononuclear fraction as a whole. These data provide compelling evidence that 17-AAG may be effective in the treatment of *c-kit*-related diseases including mastocytosis, GIST, mast cell leukemia, subtypes of acute myelogenous leukemia, and testicular cancer.

More recently, several groups have reported that mutated B-Raf and mutated epidermal growth factor receptor (EGFR) develop strong dependence on Hsp90 and thus acquire marked sensitivity to Hsp90 inhibitors (54–56). Since B-Raf is mutated in approximately 60% of melanomas and to a lesser degree in other cancers (57), and since cells expressing mutated B-Raf appear to be dependent on its activity for their survival, Hsp90 inhibitors may have wide applicability in melanoma. Indeed, results of a clinical trial support this hypothesis (58). Similarly, the EGFR mutations described in a small percentage of non-small cell lung cancer patients also confer Hsp90 dependence and sensitivity to Hsp90 inhibitors (56). While these patients initially respond to EGFR inhibitor therapy, they almost invariably become refractory with time. However, even tumors refractory to EGFR inhibitors remain very sensitive to Hsp90 inhibitors, suggesting that Hsp90 inhibitor therapy may be an efficacious second-line therapy in these patients (56).

Hsp90 Inhibitors Target the Androgen Receptor in Prostate Cancer

Androgen receptor continues to be expressed in most hormone-independent prostate cancers, suggesting that it remains important for tumor growth and survival. Receptor overexpression, mutation, and/or post-translational modification may all be mechanisms by which androgen receptor can remain responsive to low levels of circulating androgen or to anti-androgens. Vanaja et al. have shown that Hsp90 association is essential for the function and stability of the androgen receptor in prostate cancer cells (59). These investigators reported that androgen receptor levels in LNCaP cells were markedly reduced by GA, as was the ability of the receptor to become transcriptionally active in the presence of synthetic androgen. In addition, Georget et al. have shown that GA preferentially

destabilized androgen receptor bound to anti-androgen, thus suggesting that the clinical efficacy of anti-androgens may be enhanced by combination with an Hsp90 inhibitor (60). These investigators also reported that GA prevented the nuclear translocation of ligand-bound androgen receptor, and inhibited the transcriptional activity of nuclear-targeted receptors, implicating Hsp90 in multiple facets of androgen receptor activity. Finally, Solit and colleagues have reported that 17-AAG caused degradation of wild-type and mutant androgen receptors and inhibited androgen-dependent and androgen-independent prostate tumor growth in nude mice (61). Importantly, these investigators also demonstrated the loss of Her2 and Akt proteins, two Hsp90 clients that are upstream post-translational activators of the androgen receptor, in the tumor xenografts taken from 17-AAG-treated animals.

Hsp90 Inhibitors Exert Anti-angiogenic Activity

Hypoxia inducible factor-1 α (HIF-1 α) is a nuclear transcription factor involved in the transactivation of numerous target genes, many of which are implicated in the promotion of angiogenesis and adaptation to hypoxia (for a review, see [62]). Although these proteins are normally labile and expressed at low levels in normoxic cells, their stability and activation increase several-fold in hypoxia. The molecular basis for the instability of these proteins in normoxia depends on VHL, the substrate recognition component of an E3 ubiquitin ligase complex that targets HIF-1 α for proteasome-dependent degradation (63). Hypoxia normally impairs VHL function, thus allowing HIF to accumulate. HIF-1 α expression has been documented in diverse epithelial cancers and most certainly supports survival in the oxygen-depleted environment inhabited by most solid tumors.

VHL can also be directly inactivated by mutation or hypermethylation, resulting in constitutive overexpression of HIF in normoxic cells. In hereditary von Hippel-Lindau disease there is a genetic loss of VHL, and affected individuals are predisposed to an increased risk of developing highly vascular tumors in a number of organs. This is due, in large part, to deregulated HIF expression and the corresponding up-regulation of the HIF target gene vascular endothelial growth factor (VEGF). A common manifestation of VHL disease is the development of clear cell renal cell carcinoma (CC-RCC; 64). VHL inactivation also occurs in nonhereditary, sporadic CC-RCC.

HIF-1 α interacts with Hsp90 (65), and both GA and another Hsp90 inhibitor, radicicol, reduce HIF-dependent transcriptional activity (66,67). Hur et al. demonstrated that HIF protein from radicicol-treated cells was unable to bind DNA, suggesting that Hsp90 is necessary for mediating the proper conformation of HIF and/or recruiting additional cofactors. Likewise, Isaacs et al. reported GA-dependent, transcriptional inhibition of VEGF. Additionally, GA down-regulated HIF-1 α protein expression by stimulating VHL-independent HIF-1 α proteasomal degradation (67,68).

HIF-1 α induction and VEGF expression has been associated with migration of glioblastoma cells *in vitro* and metastasis of glioblastoma *in vivo*. Zagzag et al., in agreement with the findings described in the preceding paragraphs, have reported that GA blocks

HIF-1 α induction and VEGF expression in glioblastoma cell lines (69). Further, these investigators have shown that GA blocks glioblastoma cell migration, using an in vitro assay at nontoxic concentrations. This effect on tumor cell motility was independent of p53 and PTEN status, which makes Hsp90 inhibition an attractive modality in glioblastoma, where mutations in p53 and PTEN genes are common and where tumor invasiveness is a major therapeutic challenge.

Dias et al. have reported that VEGF promotes elevated Bcl2 protein levels and inhibits activity of the pro-apoptotic caspase-activating protein Apaf in normal endothelial cells and in leukemia cells bearing receptors for VEGF (70). Intriguingly, these investigators show that both phenomena require VEGF-stimulated Hsp90 association (e.g., with Bcl2 and Apaf), and that GA reverses both processes. Thus, GA blocked the prosurvival effects of VEGF by preventing accumulation of anti-apoptotic Bcl2 and blocking the inhibition of pro-apoptotic Apaf.

Hsp90 Inhibitors Target Met and RET Receptor Tyrosine Kinases

The Met receptor tyrosine kinase is frequently overexpressed in cancer, and is involved in angiogenesis, as well as in the survival and invasive ability of cancer cells. A report by Maulik et al. (71) demonstrated a role for Met in migration and survival of small cell lung cancer (71). Met is an Hsp90 client protein, and these investigators went on to show that GA antagonized Met activity, reduced the Met protein level, and promoted apoptosis in several small cell lung cancer cell lines, even in the presence of excess Met ligand.

Hypoxia potentiates the invasive and metastatic potential of tumor cells. In an important study, Pennacchietti and colleagues reported that hypoxia (via two HIF-1 α response elements) transcriptionally activated the Met gene, and synergized with Met ligand in promoting tumor invasion (72). Further, they showed that the pro-invasive effects of hypoxia were mimicked by Met overexpression, and that inhibition of Met expression prevented hypoxia-induced tumor invasion (72). Coupled with an earlier report describing induction of HIF-1 transcriptional activity by Met ligand (73), these data identify the HIF-VEGF-Met axis as a critical target for intervention using Hsp90 inhibitors, either alone or with other inhibitors of angiogenesis. As Bottaro and Liotta (74) reported, the sole use of angiogenesis inhibitors to deprive tumors of oxygen might produce an unexpectedly aggressive phenotype in those cells that survived the treatment. These authors speculated that combination of Met inhibitors with anti-angiogenesis agents should therefore be beneficial. We suggest that combination of an anti-angiogenesis drug with an Hsp90 inhibitor would not only potentiate the antitumor effects obtained by inhibiting angiogenesis, but would also break the HIF-Met axis by simultaneously targeting both Hsp90-dependent signaling proteins.

Mutation of a related receptor tyrosine kinase, RET, is associated with human cancer and several human neuroendocrine diseases. Point mutations of RET are responsible for multiple endocrine neoplasia type 2 (MEN2A, MEN2B) and familial medullary thyroid carcinoma (FMTC). Somatic gene rearrangements juxtaposing the TK domain of RET to heterologous gene partners are found in papillary carcinoma of the thyroid (PTC; 75–77).

Possible effects of 17-AAG on RET activity and cell growth of the TT MTC cell line have been examined (78). Following treatment with 17-AAG, RET tyrosine kinase activity was inhibited by nearly 80%, as was the rate of cell growth. Thus, 17-AAG should be considered as an attractive pharmacologic agent for use as systemic therapy in patients with recurrent metastatic MTC for which nonsurgical therapy has been ineffective.

The Proteasome as An Anticancer Molecular Target

Regulated degradation of intracellular proteins is mediated by the proteasome, a 2.4-MDa molecular machine composed of approximately 60 subunits that together account for 2% of total cell protein. Proteasomes regulate the half-lives of many signaling proteins in response to environmental stimuli and rapidly degrade hopelessly misfolded proteins, which, if allowed to accumulate, can lead to apoptosis. Although the process leading from aggregation of misfolded proteins to cell death is not well understood and is likely to be multifactorial, one simple hypothesis is that accumulation of aggregated proteins results in the sequestering of numerous cellular chaperones and proteasome components in an insoluble and non-functional state, thus negatively impacting normal cell homeostasis. Deregulated protein aggregation has also been shown to cause mitochondrial membrane depolarization, release of cytochrome c, and activation of caspase cascades. Indeed, various deficiencies in proteasome processing of misfolded proteins underlie a number of neurodegenerative diseases characterized by abnormal deposition of insoluble misfolded proteins that result in the apoptotic death of neuronal cells. As described earlier in this review, the frequent acidosis and hypoxia to which cancer cells are subjected cause free radical-mediated damage to cellular proteins. If this damage cannot be repaired, such proteins are cleared from the cell via their degradation in the proteasome.

The proteasome is composed of a 20S core particle that contains three proteolytic activities that recognize hydrophobic, basic, and acidic amino acids. The 26S proteasome is composed of a 20S core particle capped on either end by a 19S ubiquitin chain recognition particle that also uses adenosine triphosphate (ATP) to unwind substrate protein, allowing it to enter the 20S core where it is degraded into small peptides two to 25 residues in length. Upon exiting the proteasome, these small peptides are rapidly degraded to their amino acid components by cytosolic peptidases. The poly-ubiquitin chain is also removed and disassembled to mono-ubiquitin for reuse. The interested reader is referred to several excellent reviews on proteasome function and on validating the proteasome and ubiquitination machinery as drug targets (79–81).

As stated previously, the 26S proteasome recognizes poly-ubiquitin chains, and most proteins destined for proteasomal degradation are first tagged (e.g., ubiquitinated) by sequential covalent addition of four or more ubiquitin moieties (a 76-amino acid, highly conserved protein present in the cytoplasm and nucleus of all eukaryotes). This process involves ATP-dependent charging of an ubiquitin-activating enzyme (E1), which then transfers ubiquitin to an ubiquitin-conjugating enzyme (E2), which in the presence

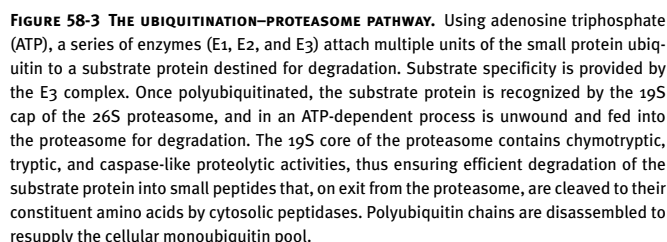


Table 58-1 Possible Mechanisms Underlying Antitumor Activity of Bortezomib

Mechanism	Comment
Inhibition of NF- κ B	NF- κ B is localized to cytoplasm by association with I κ B. On I κ B degradation by proteasome, NF κ B is able to enter nucleus and to up-regulate transcription of a number of cell adhesion, cytokine, and angiogenesis-promoting genes. However, inhibition of NF- κ B activity alone is not able to fully account for antitumor activity.
Induction of cell stress and apoptosis	Bortezomib causes accumulation of misfolded, polyubiquitinated proteins, resulting in endoplasmic reticulum stress that triggers caspase-4. Bortezomib also induces mitochondrial membrane disruption, resulting in cytochrome c release and activation of caspase-9. Bortezomib also induces activation of caspase-3 and caspase-8, independent of p53 status. Bortezomib leads to JNK activation, which may have a central role in its ability to cause apoptosis. Bortezomib cooperates with heat shock protein 90 (Hsp90) inhibitors to induce aggregation of misfolded proteins.
Anti-angiogenesis	Proteasome inhibition prevents vascular endothelial tube formation in vitro and expression of plasminogen activator, a protease involved in angiogenesis. Down-regulation of pro-angiogenic cytokines may be partially mediated by inhibition of NF- κ B.

Bortezomib is the first proteasome inhibitor to enter clinical trials in hematologic malignancies. It has shown significant activity toward multiple myeloma, and in 2005 the U.S. Food and Drug Administration (FDA) approved its use in patients with relapsed multiple myeloma. Multiple additional clinical trials are under way, examining the efficacy of this agent in various hematologic and solid tumors (82). Preliminary data suggest promising activity in mantle cell lymphoma and follicular lymphoma. Several clinical trials are also testing Bortezomib in combination with other therapeutic agents, including dexamethasone, doxorubicin, melphalan, and Hsp90 inhibitors. For an in-depth review of Bortezomib's possible mechanism(s) of action and clinical evaluation, the reader is referred to an excellent review by Roccaro et al. (82). A summary of the possible mechanisms underlying the antitumor activity of Bortezomib is shown in [Table 58-1](#). Given the focus of the current narrative on Hsp90 and the proteasome, the following section summarizes the rationale for combined use of inhibitors of the proteasome and Hsp90.

Proteasome-mediated degradation is the common fate of Hsp90 client proteins in cells treated with Hsp90 inhibitors (83,84). Proteasome inhibition does not protect Hsp90 clients in the face of chaperone inhibition—instead client proteins become insoluble (42,85). Since the deposition of insoluble proteins can be toxic to cells (86,87), interest has arisen in combining proteasome inhibition with inhibition of Hsp90, the idea being that dual treatment will lead to enhanced accumulation of insoluble proteins and trigger apoptosis. This hypothesis is particularly appealing given the clinical efficacy of Bortezomib alone (82). Initial experimental support for such a hypothesis was provided by Mitsiades

et al. (88), who reported that Hsp90 inhibitors enhanced multiple myeloma cell sensitivity to proteasome inhibition. Importantly, transformed cells are more sensitive to the cytotoxic effects of this drug combination than are nontransformed cells. Thus, 3T3 fibroblasts are fully resistant to combined administration of 17-AAG and Bortezomib at concentrations that prove cytotoxic to 3T3 cells transformed by HPV16 virus encoding viral proteins E6 and E7 (89). In the same study, Mimnaugh et al. demonstrated that the endoplasmic reticulum is one of the main targets of this drug combination. In the presence of combined doses of both agents that show synergistic cytotoxicity, these investigators noted a nearly complete disruption of the architecture of the endoplasmic reticulum. Since all secreted and transmembrane proteins must pass through this organelle on their route to the extracellular space, it is not surprising that a highly secretory cancer such as multiple myeloma would be particularly sensitive to combined inhibition of Hsp90 and the proteasome. One might speculate that other highly secretory cancers, including hepatocellular carcinoma and pancreatic carcinoma, would also respond favorably to this drug combination.

Additional Rationales for Inhibiting the Ubiquitin–Proteasome System in Cancer

As stated previously, the proteasome inhibitor Bortezomib has demonstrated activity as a single agent in multiple myeloma and in other hematologic malignancies. While general interference in the clearance of misfolded proteins is likely to be a major contributor to the efficacy of this agent, other more specific effects of proteasome inhibition should also be considered (82). In these hematologic cells, the transcription factor NF- κ B plays a particularly important role. Not only does it inhibit apoptosis, but it actively up-regulates transcription of growth factors such as interleukin-6 and angiogenic factors such as VEGF. As a transcription factor, the activity of NF- κ B requires nuclear entry. This in turn is regulated by targeted, proteasome-mediated degradation of I κ B, a protein that interacts with NF- κ B and restricts it to the cytosol. Treatment of cells with Bortezomib has been shown to prevent the degradation of I κ B, thus resulting in retention of NF- κ B in the cytosol.

Other important tumor suppressor proteins degraded by the proteasome include p53 and p27. Thus, proteasome inhibition promotes the accumulation of these proteins. Investigators have identified more specific approaches to prevent inappropriate p53 and p27 degradation by searching for inhibitors of the E3 ligases that recognize these proteins. The E3 interacting with p53 is termed “MDM2” (double-minute protein 2). Two small molecules that interfere with MDM2/p53 interaction have been identified (90,91). While mechanistically distinct, these agents both result in accumulation of wild-type p53 in tumor cells in vitro and shrink tumors growing in mice. The tumor suppressor p27 is degraded by the E3 ligase SKP2 (S-phase kinase-associated protein 2). SKP2, which also targets other antiproliferative molecules in the cell including the retinoblastoma family protein p130, the cyclin-dependent kinase inhibitors p21 and p57, and the inhibitory transcription factor FoxO (forkhead box protein O) is overexpressed in many human cancers (80). Furthermore, molecular knockdown of

SKP2 using RNA interference techniques or intracellular injection of SKP2-specific antibodies slows the proliferation of cancer cells in vitro (80,92). Thus, SKP2 is a valid therapeutic target in its own right, and several pharmaceutical companies have established drug discovery programs specifically targeting this ubiquitin ligase.

Why Are Tumor Cells Uniquely Sensitive to Hsp90 Inhibition and Proteasome Inhibition?

It is apparent, from both preclinical and clinical observations, that Hsp90 inhibitors can be administered in vivo at doses and schedules that significantly impact tumor growth but display minimal target-related toxicity in normal tissues or in the whole organism. This is the case for several small-molecule inhibitors, including 17-AAG and 17-DMAG, the synthetic purine mimetic PU24FCL, and a novel peptidomimetic inhibitor of the N-terminal Hsp90 nucleotide binding site, shepherdin (58,93–96). Since Hsp90 is highly expressed in most, if not all normal tissues, these findings require an explanation. Indeed, when murine model systems are examined in vivo, Hsp90 inhibitors are found to concentrate in tumor tissue, while being rapidly cleared from normal tissue with a half-life similar to that of drug in plasma (58,93,95,96). The Hsp90 inhibitor 17-AAG also has been reported to actively concentrate in tumor cells in vitro (97).

Since preferential accumulation of these Hsp90 inhibitors in tumor versus normal tissue may provide the observed therapeutic (or at least biologic) index, it is important to understand the reason for this phenomenon. A possible explanation put forth by Kamal and colleagues suggests that enhanced drug binding to tumor cell Hsp90 reflects the activity state of the Hsp90 chaperone machine in tumor versus normal cells (98). They proposed that enhanced the ATPase activity of the chaperone in tumor cells, which is dependent on preferential recruitment of Hsp90 to a multicomponent chaperone complex, is responsible for the increased affinity of Hsp90 inhibitors in tumor cells.

Others have reported that expression of NAD(P)H: Quinone Oxidoreductase I (NQO1), also known as DT-diaphorase, dramatically enhances cellular sensitivity to 17-AAG (58,99). NQO1 generates the hydroquinone version of 17-AAG, which has been reported to bind more tightly to Hsp90 when compared with 17-AAG itself (100). Further, the presence of NQO1 in a cell seems also to lead to increased total accumulation of intracellular ansamycin molecules, presumably reflecting the increased water solubility of the 17-AAG dihydroquinone and its decreased propensity to cross membranes. Thus, by this model, NQO1 serves to trap 17-AAG in cells while simultaneously enhancing its Hsp90 binding affinity. Intriguingly, these investigators and others have shown that the presence of NQO1 in tumor cells dramatically affects cellular sensitivity to 17-AAG (58,99,100). Since high levels of NQO1 have been observed in diverse tumor types (e.g., liver, lung, colon, breast) as compared with normal tissues of the same origin (101), these data suggest an explanation for the disparate sensitivity of tumor and normal tissue to 17-AAG. However, the

similar preference of other Hsp90 inhibitors, such as the synthetic purine analog PU24FC1 and the peptidomimetic shepherdin, for tumor cells remains to be explained. Several groups are examining altered states of post-translational modification of Hsp90 in tumor versus normal cells as a possible contributing factor to this phenomenon.

The proteasome inhibitor Bortezomib also displays selective cytotoxicity toward tumor cells, both *in vitro* and *in vivo*. Why is Bortezomib not more toxic to normal cells? While there may not be a simple answer to this important question, Kisselev et al. (102) have shown that, at therapeutic doses of Bortezomib *in vivo*, and following the intermittent schedule of administration approved for patients, only the chymotryptic activity of the proteasome is significantly inhibited, but the overall rate of protein degradation is reduced by less than 40%. Because cancer cells may require their proteasomes to be at full capacity to handle the load of continually generated misfolded proteins, any reduction in proteasome activity, even for a brief period, may prove fatal. In contrast, normal cells may be able to tolerate proteasome function at 50% of capacity for an extended time. Some of our data described earlier in this review supports this hypothesis. In the preclinical

studies in which we observed dramatically enhanced toxicity (and dramatically enhanced insoluble ubiquitinated protein deposition) by combining low-dose Hsp90 inhibitors with Bortezomib, we found that maximal benefit of the combination required only a 50% reduction in proteasome activity (89).

Conclusion

The proteasome and Hsp90 together comprise approximately 4% of total cellular protein, and separately and in concert, they regulate critical mechanisms responsible for maintaining cellular homeostasis in the face of environmental change. As such, they certainly can be considered to be "housekeeping" proteins. Nonetheless, it should be clear from this brief overview that both proteins are proving to be exciting and clinically relevant anticancer molecular targets. The unexpected finding that cancer cells are more sensitive to interdiction of the protein degradation and chaperoning machinery than are normal cells suggests that further emphasis on these pathways as drug targets can provide additional therapeutic strategies to attack this disease.

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Cancer Chemoprevention

Chemoprevention is the use of pharmacologic interventions to reduce the risk of cancer or to treat or reduce the risk of intraepithelial neoplasia (IEN; 1). As a noninvasive lesion representing an often pathologically discernable intermediate state between normal and malignant tissue, IEN has a substantial cancer risk (2). Used in therapy drug development for some time, molecular targeting has become a mainstay of chemopreventive drug development as well (3). The molecular biology of preinvasive carcinogenesis and drug interventions was substantially advanced by translational research of a few pioneering groups, including studies by Hong and colleagues in head and neck carcinogenesis and its response to retinoids (1,4–8). These early studies presaged the emergence of molecular-targeted approaches, the major focus of chemopreventive drug development today.

Molecular-Targeted Prevention

Molecular-targeted drug development is based on the concept that neoplasia is a multistep process, which involves accumulating genetic and epigenetic alterations driven by genomic instability, and a multifocal process, which involves field carcinogenesis and clonal spread (1,9). Hallmarks of these processes include evasion of apoptosis, self-sufficiency in growth signals, insensitivity to antigrowth signals, strong replicative potential, and sustained angiogenesis. These molecular alterations and hallmarks can develop in IEN. Major advances in molecular-targeted chemoprevention include the drugs tamoxifen and raloxifene (targeting the estrogen receptor [ER]), finasteride (targeting 5 α -reductase), and celecoxib (targeting cyclooxygenase-2 [COX-2]; 10–14).

Biomarkers play a major role in all aspects of molecular-targeted chemoprevention, including as (1) molecular targets for identifying new agents; (2) cancer risk, prognosis, or predictive markers for selecting (or stratifying) study patients; (3) targets to help determine the tissue delivery of biologically active doses; (4) endpoints of phase 2 drug activity trials; and (5) surrogate efficacy and toxicity endpoints in phase 3 cancer prevention trials. Although receiving keen interest, surrogate biomarkers are extremely complicated and may yet be a long way from validation as primary endpoints of phase 3 trials. Encouraging results on

potential new surrogate endpoint biomarkers are emerging from work on proteomic and genomic profiling in trials of the selective COX-2-inhibitor celecoxib (15,16), highlighting the convergence of molecular markers in chemoprevention with those of early-detection research. Molecular biomarkers also can be used to confirm IEN response, the importance of which was suggested by genetic abnormalities that persisted at the site of head and neck IEN that had responded completely (clinically and histologically) in a chemoprevention trial (17).

Molecular biomarkers also are used in the emerging field of preventive pharmacogenomics. Germ-line *BRCA2* mutations in people receiving tamoxifen, *SRD5A2* polymorphisms in people receiving finasteride, cyclin D1 polymorphisms in people receiving retinoids, *CYP2C9* genotypes in people receiving nonsteroidal anti-inflammatory drugs (NSAIDs), and epidermal growth factor receptor (*EGFR*) tyrosine kinase (TK) domain mutations in patients receiving *EGFR* TK inhibitors (TKIs) are important examples of pharmacogenomic biomarkers (18–20).

Prevention–Therapy Convergence

Molecular-targeting research is blurring the distinction between malignancy and premalignancy and between cancer therapy and prevention. A new generation of targeted drugs with acceptable therapeutic indices for prevention and therapy is emerging from the molecular study of neoplasia (IEN and cancer), drug effects on relevant pathways, and cancer risk/prognosis (3). Targeted drugs can move from therapy to prevention (exemplified by tamoxifen, aromatase inhibitors, and *EGFR* inhibitors) or vice versa (celecoxib). It is likely that tamoxifen both prevented and treated subclinical cancer in the Breast Cancer Prevention Trial (BCPT) and adjuvant breast cancer trials. Always problematical, the distinction in cancer survivors between a second primary tumor (SPT), which is a prevention endpoint, and recurrence, which is a therapy endpoint, has been blurred further by molecular studies in breast and head and neck neoplasia. The distinction between cancer and IEN is blurred in definitively treated oral cancer patients who develop IEN at a very high risk of a new cancer because of loss of heterozygosity (LOH; 21). Furthermore, it is very difficult to determine if the

new cancer developing in these patients is an SPT or a recurrence. Rigorous clinical determinations of SPT or recurrence following curative treatment of head and neck cancer have been questioned by genetic profiling that revealed substantial molecular ambiguity regarding the origins of the subsequent cancers. For example, over 50% of the clinically defined SPTs were molecularly determined to be recurrences (i.e., to have genetic profiles consistent with clonal spread of the original tumor; 22).

Convergent Trial Designs

Two convergent trial designs involving molecular-targeted agents are (1) a phase 1 design in which toxicity and pharmacodynamic effects (e.g., optimal biologic doses) are assessed to determine the dose of an agent for subsequent phase 2 testing in either prevention or therapy and (2) a therapy design with imbedded prevention endpoints (e.g., IEN) for agents with preventive potential based on mechanistic and safety characteristics (3). The phase 1 design can include assessments of pharmacodynamic effects on tumor and surrounding or surrogate tissue. The imbedding design can include phase 2 or 3 trials in cancer settings with a prevalent IEN, for example rectal aberrant crypt foci (ACFs) in single-agent colon cancer trials. ACFs can be identified by magnifying endoscopy (e.g., flexible sigmoidoscopy in the rectum) and are thought to be precursors of adenomas. ACFs often show APC loss, *K-ras* mutations, and EGFR and erbB2 up-regulation, and the number, size and dysplastic features of ACFs correlate with the number of adenomas. ACFs appeared to be suppressed by NSAIDs in observational and by EGFR TKIs in preclinical studies (23). Another imbedded convergence approach is to assess at-risk tissue in adjuvant trials, for example bronchoscopic studies in adjuvant lung cancer trials. A recent study detected EGFR TK domain mutations in histologically normal lung tissue surrounding a primary lung adenocarcinoma with EGFR mutations (24). This apparent field effect raises important biologic issues and may help identify patients more likely to benefit from adjuvant therapy with EGFR TKIs. Drug activity in high-risk IEN is relevant to the therapy setting; prevention trials in high-risk settings and therapy trials have similar sizes, durations, costs, and ethical considerations (high cancer risk justifies potential adverse drug effects, as does cancer prognosis; 25). Highest-risk IENs, such as familial adenomatous polyposis and oral IEN with LOH, are promising settings for convergent drug development.

Short-term trials in patients before a scheduled surgery also can be used for early-phase convergent drug development, as illustrated by recent studies of EGFR TKIs in breast neoplasia. The EGFR TKIs gefitinib and erlotinib reduced cell proliferation in randomized presurgical trials in women with ductal carcinoma in situ or early-stage breast cancer (26). Although not involving therapy, a novel convergent approach is to imbed prevention endpoints in a screening study. An ongoing randomized trial of inhaled budesonide is imbedded within a spiral computed tomography (CT) screening study involving high-risk people with peripheral lung nodules (presumed precursors of adenocarcinoma). Based on strong preclinical data and a provocative secondary analysis of a randomized controlled trial (RCT; 27), this novel trial is the first formal clinical assessment of preventive effects on adenocarcinoma precursors in the peripheral airway.

Promising Convergent Targets and Drugs

Many promising targets for cancer prevention and therapy are in preclinical studies related to drugs currently in clinical testing (Table 59-1). Some of the major signaling pathways with promising molecular targets are discussed in the following sections.

EGFR Signaling

EGFR is upstream of several major targets/pathways, including COX-2, PI3K, and vascular endothelial growth factor (VEGF), and has complex interactions with retinoic acid signaling and the IGF axis (discussed later). (EGFR also is upstream of cyclin D1, signal transducer and activator of transcription-3 [STAT3], and

Table 59-1 Molecular Targets and Their Agents in Development for, or Relevant to, Cancer Prevention and Therapy

Molecular Targets	Agents
Prevention and Therapy	
ER-α ^a	Tamoxifen, ^a raloxifene, ^b arzoxifene
5α-reductase ^b	Finasteride, ^b dutasteride
COX-2 ^a	Celecoxib, ^a rofecoxib
Ornithine decarboxylase	DFMO
p53	INGN201, ONYX-015
5-LOX	Zileuton
Prostacyclin	Iloprost
Aromatase	Exemestane, letrozole, anastrozole
Androgen receptor	Flutamide
PPAR-γ	Rosiglitazone
Retinoic acid receptor/ retinoid X receptor	9- <i>cis</i> -retinoic acid
Retinoid X receptor	Targretin
EGFR	Gefitinib, erlotinib, cetuximab
Therapy^c	
Farnesyl transferase	Tipifarnib, lornafinib
mTOR	RAD-001, CCI-779
DNA methyltransferase	Azacitidine
Histone deacetylase	SAHA
P13K/Akt	Deguelin, myo-inositol
MMP	Marimistat (broad), matlystatin B (MMP-1), metastat (MMP-2/9)
TRAIL	Apo2L/TRAIL
CDK	Flavopyridol (cdks 4/6,2,1); BMS 387032, seliciclib (cdks 2,1)
Her-2	Trastuzumab
VEGF	Bevacizumab, VEGF trap
VEGFR	Sorafenib, sunitinib, AZD2171, ZD6474, AMG 706, PTK 787
PDGFR	Imatinib, sunitinib, AZD2171, PTK 787
C-KIT	Imatinib, sunitinib, AZD2171, PTK 787
RET	ZD6474, sunitinib, sorafenib, AMG 706
IGF-1R	CP751871, A12, IGF1R3
FGFR	BIBF1120, BMS 582664
MEK	AZD6244, CI-1040
B-Raf	Sorafenib
Src	Dasatinib, AZD0530
HIF-1α	17-AAG
Proteasome	Bortezomib

^a Target and agent involved in U.S. Food and Drug Administration–approved cancer risk reduction or IEN treatment.
^b Target and agent involved in established cancer-risk reduction/chemoprevention.
^c Therapy targets and agents with potential for chemoprevention.
5-LOX, 5-lipoxygenase; CDK, cyclin-dependent kinase; DFMO, difluoromethylornithine; EGFR, epidermal growth factor receptor; ER, estrogen receptor; FGFR, fibroblast growth factor receptor; HIF-1 α, hypoxia-inducible factor-1 alpha; IGF-1R, insulin-like growth factor-1 receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MMP, matrix metalloproteinases; mTOR, mammalian target of rapamycin; PDGFR, platelet-derived growth factor receptor; PPAR-γ, peroxisome proliferator activating receptor gamma; SAHA, suberoylanilide hydroxamic acid; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Src.) The importance of EGFR as a prevention-therapy target is illustrated in lung carcinogenesis. High *EGFR* (*ErbB1*) gene copy number and protein expression occur in lung IEN and have been associated with a poor prognosis in resected non-small cell lung cancer, and EGFR inhibitors have activity in a mouse lung cancer prevention model and in non-small cell lung cancer therapy (in association with high EGFR). *EGFR* TK domain mutations (which are associated with EGFR TKI response) have been detected in high-risk nonmalignant lung tissue (24). EGFR is a potential target for convergent drug development in several sites and, as discussed in the following paragraphs, EGFR signaling has complex pathway interactions and feedback loops that make it very promising for use in combination targeting approaches.

Polyunsaturated Fatty-Acid Metabolic Signaling

Arachidonic and linoleic n-6 polyunsaturated fatty acid metabolic pathways harbor many promising new targets. COXs and lipoxygenases (LOXs) mediate the oxidative metabolism of these fatty acids into the bioactive lipids prostaglandins (PGs), hydroxyicosatetraenoic acids, and hydroxyoctadecadienoic acids (HODEs, including 13-S-HODE). Growing evidence indicates that there is a dynamic balance between enzymes involved in the metabolism of linoleic acid and arachidonic acid. Recent evidence suggests that certain of these enzymes and their metabolic products within neoplastic cells and their microenvironment have both pro- and antitumorigenic effects (28,29). The arachidonic acid pathway harbors the protumorigenic enzymes COX-2, 5-LOX, and 12-LOX (a dual inhibitor of 5- and 12-LOX is in development). COX-2 is a well-studied target of NSAIDs, and new targets downstream of COX-2 include prostacyclin and, at the level of PGE₂, the PGE receptors, microsomal PGE synthase-1, and 15-hydroxyprostaglandin dehydrogenase. The linoleic acid pathway harbors the antitumorigenic enzyme 15-LOX-1 (which converts linoleic acid to 13-S-HODE) and other promising new targets (e.g., GATA-6, protein kinase G, and PPARs) involved in a recently discovered apoptotic signaling pathway (30). Losses of 15-LOX-1 expression and enzymatic activity were the only significant changes in LOX metabolism that related to the loss of cell differentiation and apoptosis in colon cancer cells in vitro and in polyps of familial adenomatous polyposis patients (31). Pharmacologic or genetic restoration of 15-LOX-1 induces apoptosis and suppresses tumorigenesis in vivo. 15-LOX-1 interacts with GATA-6, protein kinase G, histone deacetylase (HDAC), and methyltransferase (upstream 15-LOX-1 regulators) and PPAR- δ and PPAR- γ (downstream 15-LOX-1 mediators) to induce apoptosis and suppress carcinogenesis (31–33). 13-S-HODE down-regulates PPAR- δ to activate PPAR- γ and induce apoptosis, indicating that polyunsaturated fatty acid oxidative metabolism can influence the balance between PPAR- δ and PPAR- γ .

Retinoic Acid Signaling

Promising convergent targets also are emerging from studies of retinoid signaling through retinoic acid receptor (RAR) and retinoid X receptor (RXR) types, subtypes, and isoforms (1,4). Retinoids modulate cell growth and gene expression by activating nuclear RARs and RXRs, each of which exists in several isoforms

and possesses distinct functions. For example, RAR- β_2 subtype is a putative tumor suppressor, whereas RAR- β_4 has oncogenic properties. RAR- β_2 suppresses COX-2 expression and frequently is methylated in tobacco-related and other neoplasias. Recent studies have identified a novel RAR- β_2 -induced gene, *RRIG1*, which encodes a cell membrane protein that binds to and inhibits RhoA activity and mediates the effects of RAR- β_2 on cell growth and gene expression (34). These findings highlight a novel molecular pathway involving RAR- β_2 , *RRIG1*, and RhoA, all of which are promising convergent targets.

IGF Axis

Targeting the insulin-like growth factor (IGF) axis also is a promising approach for both prevention and therapy, as illustrated by recent data in the aerodigestive tract. Elevated levels of IGF-1 and reduced levels of IGF binding protein 3 (IGFBP-3) are associated with increased risk and poor prognosis in lung and other cancers; IGF-1 is a mitogen for a number of neoplastic cells types. The IGF-1 receptor (IGF-1R) is activated during lung carcinogenesis in vitro and in vivo in animals. Targeting IGF-1R and its downstream pathways (e.g., by the use of IGFBP-3) inhibits survival of premalignant and malignant bronchial epithelial cells and vascular endothelial cells, decreases tumor growth and angiogenesis, and so may be effective for cancer chemoprevention and therapy (35).

PI3K/Akt Signaling

Targeting the PI3K/Akt signaling pathway is another promising approach, especially in the lung. Tobacco carcinogens induce Akt activation and lung carcinogenesis. The Akt pathway is activated in bronchial premalignancy (both proximal airway and alveolar epithelium) in smokers and patients with lung IEN or cancer. Preclinical in vivo studies show that deguelin and myo-inositol have preventive activity in lung tumorigenesis, in part via suppressing the PI3K/Akt pathway (36,37). The kinase mammalian target of rapamycin (mTOR) is downstream of Akt, and the mTOR inhibitor CCI-779 blocked malignant progression of premalignant lesions with activated mTOR arising in the alveoli of mice that develop lung cancer because of activated K-ras (38). The mechanism by which CCI-779 inhibited tumorigenesis was unexpected. These lesions were infiltrated with macrophages, shown immunohistochemically to have prominent activation of mTOR signaling. A similar pattern of macrophage infiltration occurred in human alveolar premalignant lesions (atypical alveolar hyperplasia). Treatment with CCI-779 induced apoptosis of macrophages, which coincided with the chemopreventive effect. In vitro, CCI-779 had no effect on LKR-13, a lung adenocarcinoma cell line derived from this mouse, whereas it did induce apoptosis of macrophages, and conditioned media from macrophages directly stimulated the proliferation of LKR-13 cells. In summary, mTOR is activated in lung premalignancy and is required for malignant progression in the lung. This kinase drives tumorigenesis in part through macrophages, a prominent component of the tumor microenvironment, and the antitumor effect of mTOR inhibition required the presence of the tumor microenvironment. These findings have two important implications: mTOR is a potentially important kinase target, and the tumor microenvironment is

crucial in malignant progression and a source for novel targets in chemoprevention. An mTOR inhibitor also has reversed Akt-dependent prostatic intraepithelial neoplasia in transgenic mice (39).

The Angiogenic Switch

The angiogenic switch is a target within neoplastic cells, their endothelium/microenvironment, or bone marrow-derived cells recruited to the neoplastic site (40–43). Drugs targeting various VEGF receptors (VEGFR including VEGFR1–3), chemokine receptors (CXCR-2 and CXCR-4), and matrix metalloproteinases (MMP including MMP9), which can regulate the angiogenic switch, are under clinical development in therapy and early development for prevention.

Stem Cells

Targeting bone-marrow–derived stem cells for cancer prevention is an area of intense new interest. These stem cells are recruited to neoplastic sites by chronic inflammation and tissue injury, as demonstrated by elegant studies within *Helicobacter (H.) pylori*–induced gastric cancer (44). Many factors regulate these stem cells, including cytokines and chemokines (such as interleukin-1β), and so agents targeting inflammation may influence the mobilization or engraftment of circulating stem cells.

Off-Target Effects

Developing targeted drugs for prevention involves a number of complicated signaling pathways and off-target effects. Drugs associated with a major target can have effects on known and probably unknown other targets. For example, the preventive activity of so-called COX-2–selective NSAIDs may be mediated via molecular targets other than COX-2, such as PKG, GATA-6, 15-LOX-1, and PPARs. IGFBP3s (including IGFBP-3) can inhibit the growth of neoplastic cells by both IGF-1R–dependent and –independent mechanisms. Complex positive and negative interactions and cross-target effects within these and other signaling pathways are candidate targets for new approaches, including combinatorial targeting (1,3,25,28–30,33,34 45,46).

Combinations and Multiple Targets

Preclinical prevention-related data support many promising molecular-targeted combinations with potential independent plus interactive effects. Several promising combinations are listed in Table 59-2, and data supporting a few of these combinations are cited here. Methyltransferase inhibitors plus histone deacetylase (HDAC) inhibitors have been shown to be highly active in vitro and in suppressing lung tumorigenesis in vivo. Combining an IGF-1R inhibitor with an EGFR inhibitor is based on the ability of IGF-1R to heterodimerize with EGFR, thus inducing protein expression involved in cell survival and interfering with the antitumor activity of EGFR TKIs. Farnesyltransferase inhibitors can increase the apoptotic activity of IGFBP-3 in vitro and in vitro in lung carcinogenesis models (35). Many studies support COX-2–inhibitor combinations. A COX-2 inhibitor plus IGFBP-3 is based on the ability of COX-2 to suppress IGFBP-3 in lung cancer

Table 59-2 Promising Molecular-Targeted Combinations for Prevention and Therapy

EGFR inhibitors with:	15-LOX modulators
COX-2 inhibitors	Aromatase inhibitors
RXR agonists	IGFBP-3
IGF-1R inhibitors	IGFBP-3 with:
mTOR inhibitors	FTase inhibitors
VEGF/VEGFR inhibitors	PI3K/Akt inhibitors
HDAC inhibitors	HDAC inhibitors with:
PPAR-γ agonists	DNMT inhibitors
STAT3 inhibitors	PPAR-γ agonists
COX-2 inhibitors with:	DNMT inhibitors with:
5-LOX inhibitors	RAR agonists
12-LOX inhibitors	IGFBP-3

5-LOX, 5-lipoxygenase; COX-2, cyclooxygenase-2; DNMT, DNA methyltransferase; EGFR, epidermal growth factor receptor; FTase, farnesyltransferase; HDAC, histone deacetylase; IGF-1R, insulin-like growth factor-1 receptor; IGFBP-3, IGF binding protein-3; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PPAR-γ, peroxisome proliferator-activated receptor-γ; RAR, retinoic acid receptor; RXRs, retinoid X receptors; STAT3, signal transducer and activator of transcription-3; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

cells. COX-2 inhibitors plus EGFR inhibitors (e.g., in the colon) or aromatase inhibitors (in the breast) involve positive feedback loops (45,46). COX-2 inhibitors block prostaglandin activation of EGFR and induction of aromatase, possibly allowing lower doses and less toxicity of each agent. COX-2 inhibitors (and HDAC inhibitors) can increase the sensitivity of lung cancer cells to EGFR inhibitors (possibly via effects on the epithelial-to-mesenchymal transition). COX-2 inhibitors plus EGFR TKIs almost completely prevented adenoma development in Apc/min mice (47).

Biomarker Cancer Risk Models

The identification of high-risk IEN is a major priority for targeted preventive drug development. This work can include the identification of molecular aberrancies in exfoliated cells and the use of novel imaging technologies. Genetic instability and clonal selection create the risk of cancer development and can be marked by LOH. The 3-year oral cancer risk of oral IEN with LOH at 3p14 and/or 9p21 is 25% (6,48–51). Genes implicated in this cancer risk include the FHIT tumor suppressor gene (found at 3p14) and the p16/p15/p14 tumor suppressor genes (found at 9p21). The cancer risk increases to at least 35% with the addition of LOH at any other site of a known or candidate tumor-suppressor gene (e.g., TRAIL-R1 and TRAIL-R2 at 8p21 and p53 at 17p13, respectively). The cancer risk associated with LOH in oral IEN has been confirmed by the consistent results of three independent groups. LOH at 3p and/or 9p in IEN associated with curatively treated oral cancer has a 69% risk of a new oral cancer in 3 years. There is a biomarker-based model for the oral cancer risk of oral IEN that integrates p53, LOH and chromosomal polysomy (52). A preliminary report outlines similar biomarker panel for the cancer risk of esophageal IEN (Barrett’s esophagus; 53).

Novel risk assessment models are emerging from the joint efforts of neoplasia biology (e.g., to identify somatic genetic alterations) and molecular epidemiology (to identify constitutional genetic alterations). This work demonstrates that studies of a single gene or signaling pathway can identify germ-line polymorphisms for assessing risk and carcinogen susceptibility and can 2x epigenetic or genetic events for early detection and prognosis. These studies can also help in understanding the mechanisms of preventive drug response or resistance. Genes first explored for aberrations in tumors have been explored later for germ-line aberrations contributing to cancer risk and vice versa, providing new targets for cancer prevention. For example, germ-line type-II 5AR gene (*SRD5A2*) alterations have been associated with cancer risk, somatic *SRD5A2* mutations have been associated with carcinogenesis, and the *SRD5A2* protein is the target of finasteride for preventing prostate cancer (12).

Chemoprevention Trials

Nonmolecular-Targeted Trials

Although too toxic for long-term use high-dose isotretinoin provided the proof of principle of chemoprevention in a randomized trial showing that it prevented second primary cancers in patients after definitive treatment of a first head or neck cancer (54,55). A subsequent trial of low-dose isotretinoin unfortunately did not reduce second primary cancers, recurrences, or mortality (56). These nontargeted trials did help develop many potential targets discussed previously (Promising New Convergent Targets and Drugs). Despite consistent epidemiologic data suggesting that β -carotene is associated with a lower risk of lung cancer, β -carotene supplements alone or combined with vitamin A or E increased lung cancer risk in two RCTs in nearly 50,000 smokers and/or asbestos workers (57,58). These RCTs suggested that harm correlated with smoking intensity: No evidence from RCTs indicated that β -carotene increased lung cancer risk in nonsmokers, former smokers, or moderate (less than one pack per day) smokers. Notwithstanding the generally negative results with vitamin and mineral supplements in developed countries, the combination of β -carotene, vitamin E, and selenium significantly reduced the incidence of gastric cancer and all cancers as well as mortality from cancer in a randomized trial involving 29,581 people in Linxian, China, possibly reflecting the restoration of healthful nutrient levels in an undernourished population (59).

The only proven chemopreventive approach for Barrett's esophagus is photodynamic therapy (PDT). A recent RCT found that PDT doubled the rate of regression of high-grade disease to a lower grade of dysplasia or normal-appearing epithelium (77% vs. 39%) and halved the rate of cancers (13% vs. 28%) at 2 years (60). The PDT group had a rate of adverse events of 94% (vs. 13% in control group). Complications of PDT in this trial included mild phototoxicity (68%) and significant stricture formation (36%) as well as vomiting, chest pain, constipation, and pyrexia. In mid 2003, the U.S. Food and Drug Administration (FDA) approved and granted orphan drug designation to a photosensitizing porphyrin mixture (Photofrin) in conjunction with PDT for ablation

of high-grade disease in patients with Barrett's esophagus who are unable or unwilling to undergo esophagectomy.

Several RCTs of the NSAID aspirin presaged the COX-2-specific-targeting trials discussed in Molecular-Targeted Trials. Aspirin (325 mg/d) in 635 colorectal cancer survivors significantly reduced the number of patients with incident adenomas and caused a significant delay in the time to the first adenoma (vs. placebo; 61). An RCT of low-dose (81 mg/d) and high-dose (325 mg/d) aspirin in 1,121 patients with prior adenomas showed that only low-dose aspirin reduced the risk of adenomas (especially advanced adenomas; 62). A secondary analysis of the Physicians Health Study (PHS) (involving 22,071 U.S. male physicians) found that aspirin (325 mg every other day) for an average of 5 years did not reduce colorectal cancer risk but slightly (and nonsignificantly) reduced adenomas (63). The Women's Health Study (WHS) (involving 39,876 U.S. women) found that low-dose aspirin (100 mg every other day) for an average of 10.1 years did not reduce the risks of colorectal cancer or all or other cancers.

Calcium modestly reduced the risk of sporadic adenomas in two randomized trials but did not reduce colorectal cancer in a large-scale trial of calcium plus vitamin D (64). The incidence of colorectal cancer also was reduced by 37% in the Women's Health Initiative randomized trial of combined estrogen and progestin (vs. placebo) in 16,608 randomized postmenopausal women, but at the expense of an absolute increase in adverse cardiovascular outcomes.

Although, strictly speaking, chemoprevention involves specific compounds (including extracted or synthetic nutrients), foods in the diet also can influence the risk of cancer. Epidemiologic studies of food and diet are conflicting, and randomized trials do not indicate that reduced dietary fat is associated with a reduced risk of breast cancer or that diets high in fruits and vegetables or fiber are independently associated with reduced risk of colorectal neoplasia (65).

Molecular-Targeted Trials

SERMs and Aromatase Inhibitors

The BCPT compared the selective estrogen receptor (ER) modulator (SERM) tamoxifen (20 mg/d) with placebo in preventing breast cancer in 13,388 women at a higher-than-average risk of breast cancer. At a median follow-up of 55 months, tamoxifen reduced the incidence of invasive breast cancer by 49% and non-invasive breast cancer by 50% (10). These risk reductions were similar for all age and risk groups and were limited to ER-positive tumors. Tamoxifen also reduced the risk of osteoporotic fractures but increased the risk of endometrial cancer, thromboembolism, and cataracts. There was no effect on cardiovascular disease or mental function. Another major tamoxifen prevention trial, the International Breast Cancer Intervention Study (IBIS)-I, found a significant 32% risk reduction with tamoxifen (vs. placebo) in 7,410 randomized women (66). Tamoxifen (20 mg/d for 5 years) received supplemental approval from the FDA for reducing the risk of breast cancer in premenopausal and postmenopausal women with a 5-year predicted risk of at least 1.66%. The FDA also has approved tamoxifen to reduce the risk of contralateral cancer in breast cancer patients or of invasive breast cancer in women with ductal carcinoma in situ.

Based on suggestive breast-cancer-risk-reduction data from trials in older women to prevent or reduce osteoporosis, the Study of Tamoxifen and Raloxifene (STAR) randomly tested the SERM raloxifene (60mg/day) against FDA-approved tamoxifen (20mg/day) for 5 years for reducing the risk of invasive breast cancer and other disease outcomes in 19,747 postmenopausal women (with increased 5-year breast cancer risk). This recently completed trial showed that raloxifene was equivalent to tamoxifen in effects on invasive breast cancer, had a higher number of noninvasive breast cancers, and had fewer uterine cancers (11). The two drugs were equivalent in effects on other invasive cancer sites, ischemic heart disease events, stroke or osteoporotic fractures; and raloxifene was associated with fewer cases of pulmonary emboli and deep vein thromboses, previously FDA approved, and widely used, for the prevention and treatment of osteoporosis in postmenopausal women, raloxifene now also is FDA approved based on STAR for reducing the risk of invasive breast cancer.

Tamoxifen and raloxifene illustrate the critical issue of risk-benefit for effective, acceptable chemoprevention. Tamoxifen in BCPT reduced invasive breast cancer by 49% (noninvasive

by 50%), however, at the expense of a 2.5-fold increased risk of endometrial cancer and threefold increased risk of pulmonary embolism. Raloxifene in STAR was associated with 38% less endometrial cancer and 36% less pulmonary embolism than was tamoxifen and an equivalent low rate of invasive breast cancer in STAR. Therefore, raloxifene improves the risk-benefit profile in women at risk of invasive breast cancer. A model of these comparative risk-benefit profiles is presented in Figure 59–1. Tamoxifen had a 14.8% reduced risk overall (including cancer and severe adverse events [SAEs] vs. placebo), and raloxifene had a 29.9% reduced overall risk (including cancer, SAEs; vs. placebo; Figure 59–1D). SAEs are a major risk of chemoprevention itself, and raloxifene illustrates how to reduce this risk through reducing SAEs. Another way to counter this risk is by identifying target populations with very high cancer risks (discussed earlier in this chapter) who are more SAE tolerant.

Aromatase inhibitors produced greater reductions than tamoxifen in contralateral breast cancer incidence in adjuvant RCTs (67), with fewer endometrial cancer and thromboembolic

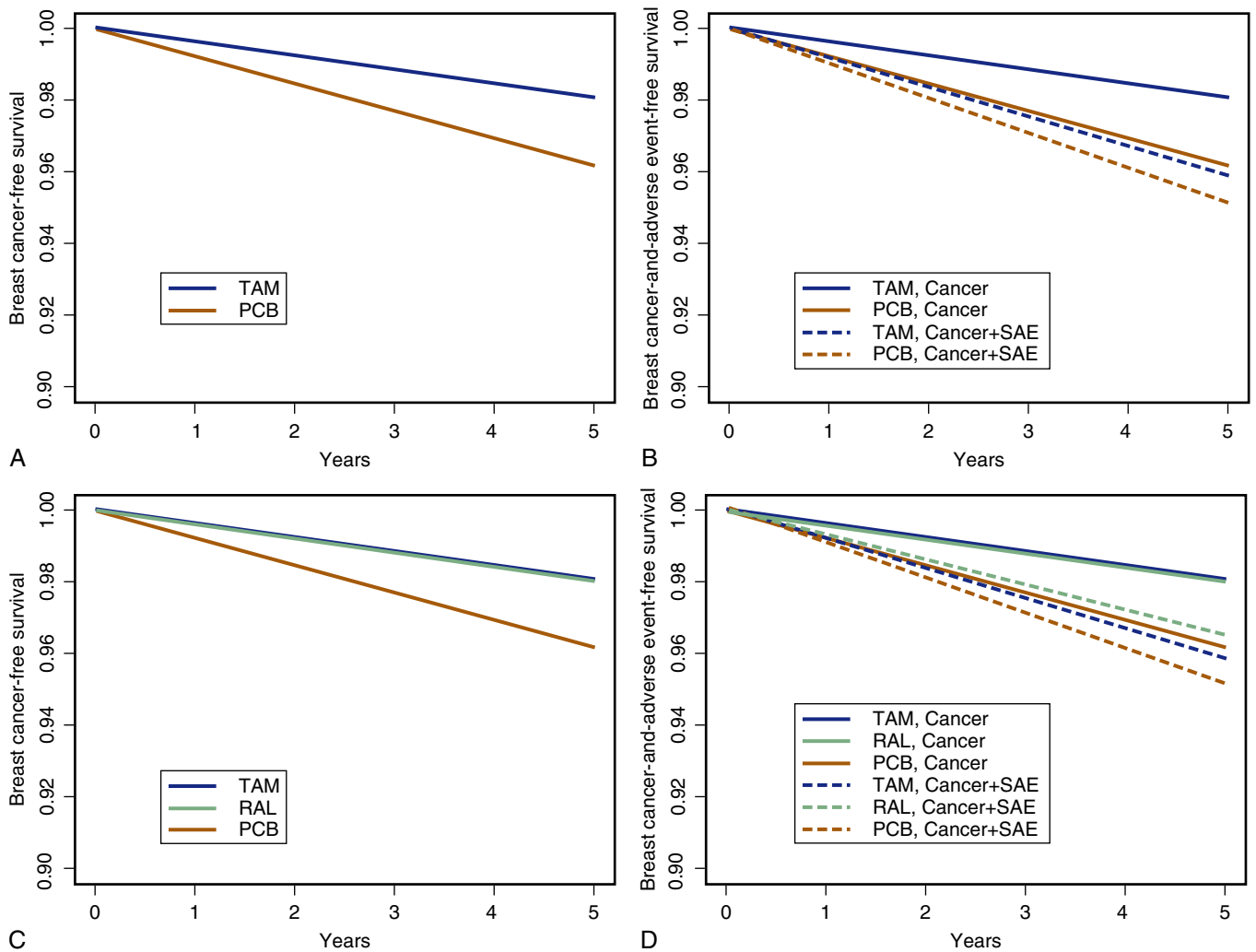


FIGURE 59-1 Risk-benefit comparisons for tamoxifen (TAM) and raloxifene (RAL) versus placebo (PCB) in breast cancer prevention. **A, B:** Comparison of TAM versus PCB without and with the consideration of severe adverse events (SAEs) including the increased risk of endometrial cancer and embolic/vascular events. **C, D:** Comparison of TAM versus RAL versus PCB without and with the consideration of SAEs. The assumed hazard rates for PCB, TAM, and RAL with the consideration of SAEs are 0.0097, 0.0082, and 0.00687, respectively. These hazard rates are based on data from the primary reports of the Breast Cancer Prevention Trial and Study of Tamoxifen and Raloxifene.

events and are under active study in prevention. Although less toxic than tamoxifen, these agents have increased risks of osteoporosis and fatal myocardial infarctions, which require further examination in long-term outcome data.

Large randomized prevention trials show that SERMs can reduce the risk of ER-positive but not ER-negative breast cancer. Although not from large randomized prevention trials, extensive data on aromatase inhibitors suggest that these agents also will prevent ER-positive and not ER-negative disease, which needs new preventive molecular-targeted approaches. Three such approaches are RXR agonists, COX-2 inhibitors, and EGFR TKIs, all of which can prevent ER-negative breast cancer in animal models (68,69).

5 α -Reductase Inhibitors

The Prostate Cancer Prevention Trial (PCPT) tested finasteride (5 mg/day), which inhibits type II 5 α -reductase, the enzyme that converts testosterone to the more potent androgen dihydrotestosterone, for 7 years (vs. placebo) in 18,882 men 55 years of age or older who had a normal digital rectal examination and prostate-specific antigen (PSA) level. Finasteride reduced the 7-year prostate cancer prevalence by 24.8% and reduced high-grade prostatic IEN and benign prostatic hypertrophy (BPH), but it also increased the rates of sexual dysfunction and high-grade prostate cancer (12). Secondary data from a trial of the dual (type I and II) 5 α -reductase inhibitor dutasteride in BPH suggested that this relative of finasteride also can reduce the risk of prostate cancer. This and other data led to an ongoing trial of dutasteride to prevent prostate cancer in men with an elevated PSA (between 2.5 and 10 ng/mL) and a negative six- to-12-core prostate biopsy. Both dutasteride and finasteride are FDA-approved for treating BPH.

The findings of finasteride in PCPT are analogous to those of tamoxifen in the BCPT and, although not placebo-controlled, STAR. Tamoxifen reduced invasive and preinvasive disease (breast) to similar degrees. It is interesting that raloxifene reduced invasive cancer to the same degree as did tamoxifen, but the rate of preinvasive breast disease was substantially higher in the raloxifene arm. Another interesting parallel between finasteride and tamoxifen is that, despite reducing overall cancer, neither reduced disease generally associated with a more aggressive phenotype (high-grade tumors in the case of finasteride; ER-negative disease, tamoxifen). The collective findings of hormonal therapy, whether in the prostate or breast, raise important clinical and biologic issues regarding the effects of this class of agents.

COX-2-Specific Inhibitors

A small RCT of celecoxib in familial adenomatous polyposis showed that high-dose celecoxib (400 mg twice daily) for 6 months reduced the number of colorectal adenomas (polyps) compared with placebo (28 vs. 4.5%, $p = 0.003$; 13). Two secondary endpoints were a reduction in polyp burden and a modest (14%) reduction in difficult-to-resect duodenal polyps. This result led to FDA approval (via accelerated approval) of celecoxib as an adjunct to standard familial adenomatous polyposis therapy. The primary

follow-up study (required by the FDA for drugs approved under the accelerated mechanism) is designed to prevent adenomas in young patients with genotypic familial adenomatous polyposis (Apc mutation carriers) but not yet expressing the phenotype. Three large placebo-controlled RCTs have found that COX-2 inhibitors significantly reduced sporadic adenomas. Rofecoxib (25 mg/day) produced a 25% reduction but also increased serious cardiovascular events, leading the manufacturer of rofecoxib to voluntarily withdraw it from the world market (70). A large RCT of celecoxib at 200 mg twice daily (b.i.d.) and 400 mg b.i.d. versus placebo found adenoma rates of 61% (placebo), 42% (200 mg b.i.d.), and 37% (400 mg b.i.d.) at 3 years (14). Another large RCT found that celecoxib at 400 mg/d reduced the 3-year cumulative rate of adenomas by 36% (vs. placebo). Both celecoxib RCTs found greater reductions in advanced (than less advanced) adenomas and celecoxib-associated increases in cardiovascular events (14,71). Celecoxib continues to be marketed worldwide, however, while being monitored carefully for cardiovascular safety.

Vaccines

Vaccinating children against hepatitis B virus (HBV) has dramatically reduced the incidence and mortality of liver cancer in Taiwan (72,73). Clinical trials have shown 85% to 95% efficacy in preventing chronic HBV infection, and this response rate can reduce the prevalence of chronic HBV infection to less than 1% in children living in HBV-endemic regions. Estimates from 2002 are that 84% of the world's countries now routinely provide the vaccine.

The FDA recently approved the quadrivalent human papilloma virus (HPV) (types 6, 11, 16, and 18) recombinant vaccine (Gardasil) for vaccination of females 9 to 26 years old for prevention of cervical cancer, cervical adenocarcinoma in situ, and high-grade cervical, vulvar, and vaginal IEN. The efficacy of the vaccine was studied in four RCTs enrolling 20,541 females (74,75). The vaccine was 100% effective for preventing HPV-16- or -18-related cervical, vulvar and vaginal IEN. This vaccine may reduce the incidence of cervical cancer and the 300,000 deaths it causes worldwide each year, not to mention the other HPV-related diseases such as oropharyngeal cancers.

Vaccines targeting *H. pylori* are also under development. The public health implications of this work are substantial in view of the fact that *H. pylori* is the major cause of gastric cancer, the fourth most common cancer and second most common cause of cancer death in the world. An estimated 50% of the world's population harbor *H. pylori* and over 335,000 deaths resulted from *H. pylori*-caused gastric cancer in 2000.

Tumor-antigen-specific vaccines are promising immunopreventive approaches (76–78). Stimulating an immune response to a specific neoplasia may last a long time and avoid the need for the extended frequent dosing required with cancer treatment or prevention drugs. Animal model studies have stimulated great interest in testing these vaccines for treating or reducing the risk of IEN and thus preventing cancer. Immunodeficient mice develop spontaneous tumors and are more susceptible than are immunocompetent

mice to carcinogen-induced tumors, revealing the importance of the host immune response for combating cancer development. Tumor-specific vaccines have demonstrated far greater activity in IEN than cancer in animals, and these vaccines are relatively inactive against advanced cancer clinically. Human host immunosurveillance for IEN is receiving strong support from recent studies in patients showing immune response against the premalignancy monoclonal gammopathy of unknown significance (a clonal expansion of transformed plasma cells that is a precursor of multiple myeloma). These data support the hypothesis that we can boost normal host immune response in IEN with vaccines, and there is intense interest in using vaccines to target IEN. HER2/neu in breast carcinogenesis and MUC1 (e.g., in pancreatic and colorectal IEN) are promising targets for convergent vaccines. Tumor-specific vaccine approaches also may be promising for treating early cancer.

Conclusion

The major direction of chemoprevention is molecular-targeted drug development, which is moving forward with advances in the biology of neoplasia (IEN and cancer), drug effects on relevant targets and pathways, and cancer risk (79,80). With apparently, in general, lesser toxicity than standard therapy drugs and greater therapeutic activity than many prevention drugs, these agents herald an era of convergent (prevention–therapy) drug development, especially in the area of advanced IEN and early cancer (81,82) (Table 59-3).

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Table 59-3 Targeted Agents with Established Cancer Risk-Reducing Effect

Intervention (Year)	Cancer Prevented
Hepatitis B vaccine (1997)	Liver cancer
Tamoxifen (1998)	Breast cancer
Finasteride (2004)	Prostate cancer
Human papillomavirus vaccine (2006)	Cervical cancer
Raloxifene (2006)	Breast cancer

Clinical oncology is expanding to the management of cancer risk (tamoxifen and raloxifene for breast cancer risk) and IEN (celecoxib for familial adenomatous polyps and tamoxifen for ductal carcinoma in situ). The future also promises to bring a substantial acceleration in the drug development process from discovery to approval, which now takes 10 to 15 years for either a preventive or therapy drug. Merging the silo of new prevention drugs with that of new therapy drugs will potentially cut overall development time in half. The loss of drugs with prevention potential because they fail in therapy testing would cease in the setting of convergent, simultaneous prevention–therapy development. Since many molecular targets or pathways altered in neoplasia also are altered in other aging-related diseases such as atherogenesis, osteoporosis, arthritis, and neurodegenerative diseases, an important new direction of molecular-targeted chemoprevention is the development of agents targeting these shared (as well as distinct) alterations in carcinogenesis and other chronic disease processes (1,81).

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